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Rapid Biochemical Technique for Phytotoxicity Modes-of-Action of Herbicides. Part I

Michigan Univ, Ann Arbor Dept of Botany

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Part I

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RAPID BIOCHEMICAL TECHNIQUE FOR PHYTOTOXICITY MODES-OF-ACTION OF HERBICIDES

Part I

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U.S. Environmental Protection Agency
Washington, D.C. 20460

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PREFACE

Phytotoxicity of pesticides is currently identified and measured by observable symptoms which are the result of biochemical disruptions at cellular levels. However, we are often unable to see a relationship between phytotoxicity and biochemical change. The physiological or physical injury, can be easily discerned and is not the subject of this research project. Rather, we are interested in studying the subject of "non-visible phytotoxicity" resulting from pesticide usage.

Basically a herbicide is a chemical which may be used to kill plant species in places where they are not wanted. Therefore most herbicides are phytotoxic substances.

Herbicides and other plant growth-regulating pesticides yield activity by virtue of their ability to interrupt or modify the basic cellular metabolism by which plant growth and development are accomplished. These behavioral responses are morphological expressions of measurable biochemical stimuli at one or more sites at the cellular level. Although laboratory methods exist for measuring the action(s) of herbicides, they are not sufficiently quantified and are not amenable to combination as one method so that the action(s) of one herbicide can be directly compared and related to another with precision.

Most cellular and biochemical measurements of pesticide effects on plants involve comparisons of tolerant and sensitive species to the effects of herbicides. Much of this data is concerned with differential detoxification and other metabolic routes of degradation; some is concerned

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with structural changes at the cellular and tissue levels. More information is needed on the sometimes subtle effects of pesticides on such fundamental cellular procedures as photosynthesis, respiration, membrane function, neucleic acid and protein metabolisms, and tissue responses such as transpiration. In view of the importance of pesticides for production of food and fiber, it is apparent that a better understanding of the biochemistry of susceptable and tolerant plants is needed. Such an understanding could lead to a rapid and reliable screening procedure to identify alternative herbicides and plant growth regulators; and a reliable method for identifying other phytotoxic pesticides at the molecular levels.

The study of cellular effects of herbicides, as well as other plant growth-regulating substances, has received increased emphasis in recent years. Generally, three important biochemical inhibitory processes have been shown to be involved in herbicide modes-of-action. These are:

1. Mitochondrial respiration, or electron transport, in plant species.
2. Protein synthesis controlled by DNA-dependent RNA.
3. Photosynthesis.

The ultimate goal of the project is to develop a method so as to identify and quantify the simultaneous occurrence of these types of inhibition in plant cells when exposed to herbicides and plant growth regulating pesticides. The method will also identify and quantify plant physiological and morphological changes in affected cells and relate these findings to types of inhibition of biochemical processes.

This new method could be applicable to the evaluation not only of herbicides but also any other biologically active chemical compounds in plant cells. I believe that the method will be a great tool to increase quality standards of foods and agricultural products which had been treated with chemicals.

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CHAPTER I

GENERAL INTRODUCTION

By Hiroshi Ikuma, Charles F. Yocum, and Robert J. Lowry

PURPOSE OF THE PROJECT

This project is designed for the development of a rapid screening technique for the localization of primary biochemical sites of action of herbicides so as to provide guidance for classification of herbicides in terms of their biochemical modes of action. The technique must detect phytotoxicity of herbicides in three important biochemical processes. They are:

1. Mitochondrial respiration, or electron transport in plant species.
2. Protein synthesis controlled by DNA-dependent RNA.
3. Photosynthesis.

In addition, the project requires the examination of physiological and morphological changes at the sub-cellular level to relate to the modes of phytotoxic action of herbicides. The technique must be rapid, reliable, and accommodate multiple, simultaneous analyses. The technique must be tested with 15 priority herbicides so as to substantiate the mode-of-action system based on related published data.

PROPOSED PLAN OF INVESTIGATION

The simultaneous analyses of the three requested cellular processes are practically impossible with the currently available techniques in the cellular, biochemical research. From studies with intact plants and isolated tissues alone, it is highly difficult to assess the phytotoxicity of herbicides at the biochemical level. If the biochemical modes of action of herbicides are emphasized as in the original request, isolated enzyme systems or organelles must be employed. This approach cannot lead us to the phytotoxicity of herbicides at the intact plant level. Although plant leaves both respire and photosynthesize, our current understanding of leaf respiration is complicated by an involvement of photorespiration which is different from mitochondrial respiration (Jackson and Volk, 1970). Frequently herbicides are effective when applied to the soil (Ashton and Crafts, 1973), suggesting that the respiratory activity of the root may be more responsible for phytotoxicity of herbicides than leaf respiration. The use of the root alone does not allow us to investigate photosynthesis, but it can enable us to study protein and nucleic acid syntheses. Ultrastructural studies need to be separated from the functional studies.

These considerations led us to propose four types of studies with isolated plant tissues; i.e., respiratory analysis of root tips, photosynthetic study with leaf disks, protein and nucleic acid syntheses in root tips, and electron microscopic analyses of root tip cells and leaf cells. As these tissue studies may not be sufficient in assessing biochemical modes of action of herbicides, we included in our original proposal the investigations with isolated organelles, mitochondria for respiratory study and chloroplasts for photosynthetic study. The investigations with isolated organelles were expected to furnish the necessary information on biochemical modes of herbicidal action, which should provide guidelines for the tissue respiration and photosynthesis work. Simultaneous measurements of oxygen and carbon dioxide were proposed for the analyses of respiration and photosynthesis with isolated tissues.

These proposed projects were approved by the Environmental Protection Agency. During the course of our investigations, it occurred in our mind that the analysis of chlorophyll fluorescence in leaves would provide invaluable information on the phytotoxic action of herbicides on leaf photosynthesis. The leaf fluorescence study was thus later added in this project. In the subsequent chapters of this report, the results from 6 different subprojects are separately reported. At the end of each chapter, our evaluation of methods employed is given.

It should be added that, in view of the emphasis of rapidity of analyses in the original request, we emphasized in our investigations to investigate early effects of herbicides. In our studies with isolated tissues, we thus examined the action of herbicides after about 2 hours of treatment. Corn root tips and soybean leaves were chosed as the major plant tissues for our studies with isolated tissues. For organelle studies, we employed the mitochondria isolated from mung bean hypocotyls and the chloroplasts from spinach, prior to the studies with corn root mitochondria and soybean leaf chloroplasts. Mung bean mitochondria and spinach chloroplasts were employed because not only of our familiarity with the isolated organelles but also they have been used more extensively in the studies of respiration and photosynthesis.

Table I-1 lists the 17 herbicides employed in our studies. This table also include the sources of herbicides. The list includes three herbicides (2,4-D, 2,4,5-T, and diuron) in addition to the original 15 herbicides, but MSMA which was on the original list was deleted because of chemical similarity to DSMA. This modification was approved by the Environmental Protection Agency.

SUMMARY OF THE RESULTS

Organelle Studies. We first established simple screening methods of herbicide phytotoxicity for the activities of both mitochondria and chloroplasts using an oxygen electrode. The methods detect possible herbicidal action on (a) inhibition of electron transport, (b) inhibition of phosphorylation, and (c) uncoupling of phosphorylation from electron transport.

Mitochondria isolated from either mung bean hypocotyls or corn roots responded to herbicides practically in an identical manner. DNOC uncoupled oxidative phosphorylation.

Table I-1. Common names, Chemical names, and sources of herbicides used in this project.

Chemical group	Common name	Chemical name	Source
Phenoxys	2,4-D 2,4,5-T	(2,4-dichlorophenoxy)acetic acid (2,4,5-trichlorophenoxy)acetic acid	Sigma EPA
Arsenicals	DSMA Cacodylate	Disodium methylarsonate Hydroxydimethylarsine oxide	Ansul Baker
Bipyridylium	Diquat	6,7-dihydrodipyrido (1,2-a:2',1'-c)pyrazinedium ion	Ortho
Carbamates	Propham Chlorpropham	Isopropylcarbanilate Isopropyl-*m*-chlorocarbanilate	K & K K & K
Thiocarbamate	Diallate	*s*-(2,3-dichloroallyl)-diisopropylthiocarbamate	Monsanto
Dinitroaniline	Trifluralin	*a*,*a*,*a*-trifluoro-2,4-dinitro-*N*,*N*-dipropyl-*p*-toluidine	Lilly
Phenol	DNOC	4,6-dinitro-*o*-cresol	Baker
Triazines	Atrazine Simazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine 2-chloro-4,6-bis(ethylamino)-*s*-triazine	Ciba-Geigy Ciba-Geigy
Triazole	Amitrole	3-amino-1,2,4-triazole	Aldrich
Ureas	Monuron Diuron (DCMU) Karbutilate	3-(*p*-chlorophenyl)-1,1-dimethylurea 3-(3,4-dichlorophenyl)-1,1-dimethylurea *tert*-butylcarbamic acid ester with 3-(*m*-hydroxyphenyl)-1,1-dimethylurea	Aldrich K & K FMC
Uracil	Bromacil	5-Bromo-3-*sec*-butyl-6-methyluracil	DuPont

Trifluralin acted to inhibit electron transport. Diallate exerted multiple effects: at low concentrations (less than 10^{-4} M), it inhibited equally the electron transport and phosphorylation; but at high concentrations (more than 10^{-4} M), it inhibited activities of ATPase and malate dehydrogenase, while it slightly stimulated the activity of cytochrome oxidase. Seven other herbicides (bromacil, chlorpropham, 2,4-D, diuron, monuron, propham, and 2,4,5-T) inhibited electron transport weakly or incompletely at the concentration range of 10^{-3} M. The remaining 7 herbicides did not affect mitochondrial activity at all at a 10^{-3} M concentration.

Chloroplasts isolated from either spinach leaves or soybean leaves responded to herbicides in an identical manner. Of the 17 herbicides tested, 4 herbicides (amitrole, cacodylate, diquat, and DSMA) affected the chloroplast activity very weakly or not at all at the concentration range of 10^{-3} M. All the rest of herbicides, with exceptions of trifluralin and diallate, inhibited photosynthetic electron transport at photosystem II; effective concentrations ranged from 10^{-7} M to 10^{-3} M depending on herbicides. Trifluralin and diallate inhibited the electron transport between two photosystems at different sites. Diallate, in addition, provided evidence that it inhibits phosphorylation at higher concentrations. At about 1,000 fold higher concentrations than their inhibition of photosystem II activity, diuron and DNOC acted to uncouple photophosphorylation. None of the 17 herbicides inhibited photosystem I activity.

Thus, most of the 17 herbicides tested were photosynthetic inhibitors, while diallate and trifluralin were equally effective in inhibiting mitochondrial and chloroplast activities. DNOC acted as an uncoupler in mitochondrial assays but as an inhibitor of photosystem II electron transport in chloroplasts; DNOC exerted these effects at the same concentration range of 10^{-5} M.

Soybean Leaf Photosynthesis. A fluorometer was constructed to measure chlorophyll fluorescence, and a method was developed to analyze both oxygen evolution and carbon dioxide uptake simultaneously with an oxygen and a carbon dioxide electrodes. The two methods posed some problems; the fluorometric determination is difficult to standardize fluorescence, while the gas analysis was hampered by large experimental variations. Leaf disks were submerged in treatment solution; this may be responsible for some of the problems we encountered.

Fluorometric analyses of herbicidal effects are still preliminary. Using diuron as the standard, the effect of 9 herbicides on the rate of fluorescence increase was examined. The results generally confirmed the chloroplast work, but the degrees of effectiveness of herbicides were not identical with the results from isolated chloroplasts. Bromacil, a potent inhibitor of photosystem II in isolated chloroplasts, induced fluorescence slowly; while cacodylate, an ineffective herbicide in chloroplast work, increased some fluorescence.

Experimental variations in gas analysis were about 35%

and the performance of the carbon dioxide electrode was suboptimal. Nevertheless, the relative effectiveness of herbicides (expressed as per cent of control) showed remarkable agreement with the results obtained from isolated chloroplasts. The agreement was qualitative. Quantitatively, the ranges of effective concentrations with leaf disks were in general higher than those with isolated chloroplasts. This decreased effectiveness of herbicides is expexpected, because the diffusion of applied herbicides to the site of action is involved in leaf disks, while the diffusion problem is much less with isolated chloroplasts. It is of interest to observe that diquat, which did not affect our chloroplast photosynthesis, acted to inhibit carbon dioxide uptake more strongly than oxygen evolution. In variant from the chlorophyll fluorescence study, bromacil was the most potent inhibitor and cacodylate gave no effect in the gas analysis.

Corn Root Tip Respiration. Using the same set-up as for gas analysis of bean leaf disk photosynthesis, the effects of 17 herbicides on respiration of isolated 3-mm corn root tips were examined. The concentration range of herbicides examined was 10^{-6} - 10^{-3} M. Experimental errors were about 10% for oxygen uptake measurements and about 20% for carbon dioxide evolution. Of the 17 herbicides tested, only three herbicides showed small effects on root tip respiration. DNOC acted as an uncoupler at 10^{-4} M, while it was slightly inhibitory at 10^{-3} M. Two phenoxy herbicides, 2,4-D and 2,4,5-T, showed slight inhibition of oxygen uptake, but not carbon dioxide evolution, at 10^{-3} M. No effect of these phenoxy herbicides was observed at lower concentrations. In view of massive damage of root tip cells caused by these herbicides at 10^{-3} M, the observed effects can be considered small. Trifluralin and diallate, which caused strong effects on respiratory activities of isolated mitochondria, were ineffective with corn root tips.

Protein and Nucleic Acid Syntheses in Corn Root Tips. An enzymatic digestion method was developed to analyze possible phytotoxic action of herbicides on protein and nucleic acid syntheses in 3-mm corn root tips. Radioactive precursors of protein, RNA, and DNA were administered to root tips in the presence or absence of a herbicide, and the uptake of these precursors into the root tips and the incorporation of precursors into protein, RNA, and DNA were examined.

With an exception of DNOC, all herbicides affected the uptake and the incorporation weakly or not at all at highest concentrations (10^{-4} - 10^{-3} M) used. DNOC inhibited both uptake and incorporation equally strongly at 10^{-4} - 10^{-5} M, the concentration range which induced uncoupling of oxidative phosphorylation in isolated mitochondria. The herbicides, which caused any inhibitory effect on the incorporation of precursors into protein, RNA, and DNA, inhibited to practically the same degree the uptake of precursors into root tips. This strong parallelism between uptake and incorporation leads us to a conclusion that none of the herbicides tested specifically inhibited protein and/or nucleic acid syntheses. The primary action of effective herbicides tested was on the uptake of precursors of respective polymers into root tips. This conclusion was supported by the observations that well-known inhibitors of protein and nucleic acid syntheses did not inhibit the uptake, but caused inhibition of specific polymer syntheses.

Electron Microscopic Studies. Each of the 17 herbicides tested induced one or more changes in the ultrastructure of the following organelles in corn root tip cells after 2 hours of treatment: mitochondria, plastids, spindle microtubules, cell wall, cell wall associated microtubules, and vacuoles. Effective concentrations differred with herbicides and organelles affected. Two most susceptible organelles for structural alteration by herbicides were spindle microtubules and mitochondria, both of which were affected by 8 herbicides. Only at 10^{-3} M, but not at lower concentrations, two phenoxy herbicides, 2,4-D and 2,4,5-T caused massive damage of the entire cell structure. None of the herbicides tested induced changes in ultrastructure of nuclei, golgi apparatus, and ribosomes significantly.

Mature soybean leaf cells were remarkably stable, in contrast to root tip cells. Only 6 of the 17 herbicides tested altered ultrastructure. Similar to root tip cells, 2,4-D and 2,4,5-T caused drastic and massive cell damage at 10^{-3} M. The other four herbicides (diallate, diquat, DNOC, and simazine) affected chloroplast structure.

Comparisons of herbicidal effect on alteration of ultrastructure of root tip cells and that of soybean leaf cells reveal that (a) leaf cells are more resistant to herbicides than root tip cells and (b) with the exceptions of 2,4-D and 2,4,5-T effects, the effective herbicides on both types of cells act differently. Apparently, young, developing cells are more susceptible to herbicides than mature, well-differentiated cells. Furthermore, since root tip cells are undergoing mitosis constantly which requires increased cellular energy,and also since the major function of leaf cells is photosynthesis, the organelles responsible for the main function of the cell appear to be more susceptible to external influence such as herbicides.

OVERALL EVALUATION

This is perhaps the largest project in herbicide research to analyze various aspects of cellular activities simultaneously at the biochemical, physiological, and morphological levels. Any one of these studies is needed to assess properly the mode of phytotoxic action of herbicides. However, for the development of a single screening method for biochemical analyses of herbicidal phytotoxicity, each approach employed in this project must be evaluated.

Organelle studies are quick and reliable, but the results do not generally reflect plant species-specific selectivity of herbicides, nor do they indicate the uptake of herbicides by the plant. Tissue level studies ingeneral show high variability in the results. The variability is likely caused by excision of the tissues under study. Theoretically, the simultaneous measurement of oxygen and carbon dioxide with a respiring or photosynthesizing tissue should lead us to reasonable conclusions to assess biochemical modes of action of herbicides. In actuality, technical problems prevented us from realization of potential of this method. In the protein and nucleic acid synthesis study with corn root tips, herbicides known to inhibit these processes (uncoupler type herbicides and carbamate type herbicides) were found to inhibit uptake of radioactive precursors by the root tips. It appears that the analyses of uptake phenomena are nore important than the analyses of protein and nucleic acid syntheses. These considerations encourage us to extend the measurements of both oxygen and carbon dioxide to include measurements of uptake of essential ions. Measurements of several parameters involved in the metabolism in a tissue will provide much more insight than organelle studies.

We consider that electron microscopic studies are important to shed further light into herbicidal action. Obviously, this approach alone cannot provide much biochemical bases of herbicidal action.

These considerations lead to a proposal that the development of a technique which utilizesmultiple analyses of involved parameters. Such a technique can be applied both for roots and for shoots.

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CHAPTER II

EFFECTS OF HERBICIDES ON MITOCHONDRIAL RESPIRATION

By Richard M. Tetley and Hiroshi Ikuma

INTRODUCTION

The basic structure and function of isolated higher plant mitochondria are similar to those of higher animal mitochondria (Ikuma, 1972). The major respiratory chain consists of flavoproteins, coenzyme Q, and b-, c-, and a-type cytochromes in sequential order from substrate to oxygen, and the respiratory chain is coupled to the phosphorylation mechanism which produces ATP from ADP and inorganic phosphate (see Lehninger, 1965). Figure II-1 illustrates the respiratory chain and associated phosphorylation.

Substrates or electron donors which feed electrons to the respiratory electron transfer chain are usually intermediates of the citric acid cycle. Succinate donates electrons directly to a flavoprotein, while NAD mediates reducing equivalents between the other substrates (isocitrate, α-ketoglutarate, malate, and pyruvate) and the respiratory chain. The chain is branched at the region of coenzyme Q towards the substrate side.

The respiratory chain has been shown to be inhibited at 4 sites by inhibitors of respiration; i.e., at the succinate-linked flavoprotein region (e.g., malonate and TTFA), at the NAD-linked flavoprotein region (e.g., amital, piericidin A), between b- and c-type cytochromes (e.g., antimycin A,

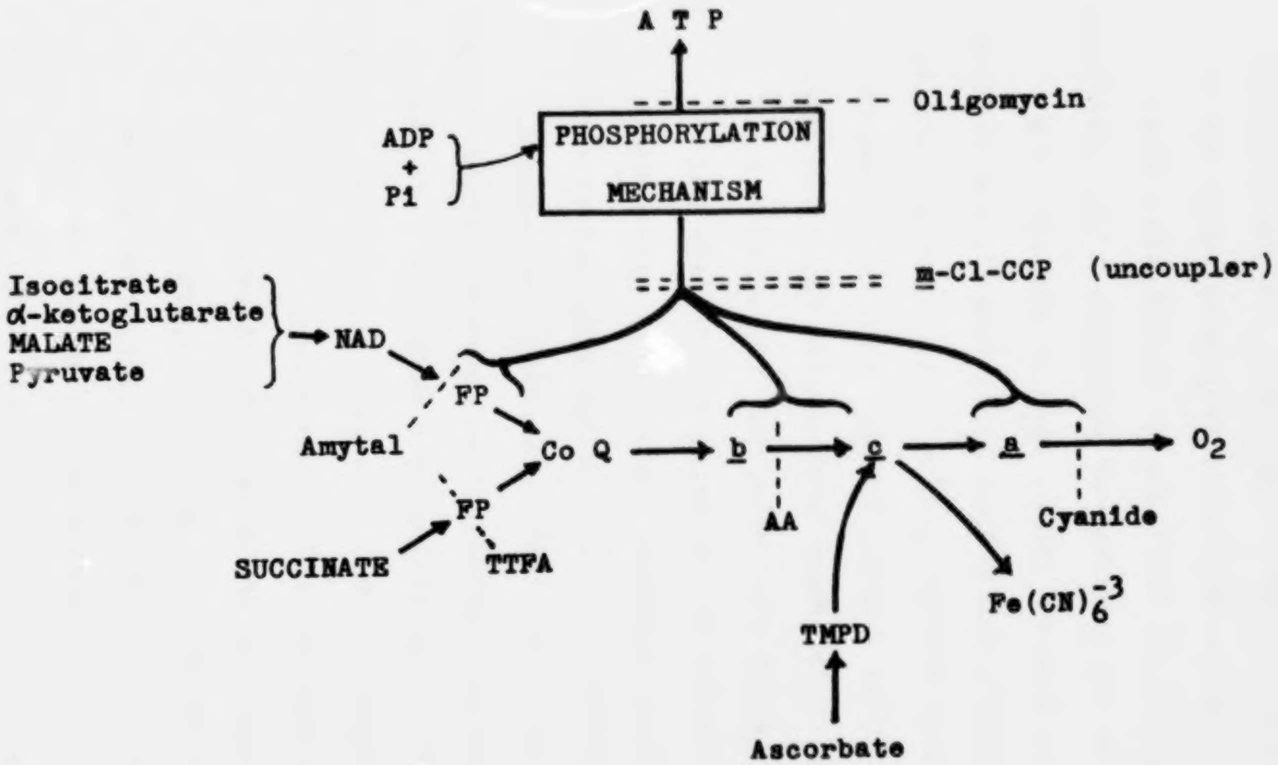

A T P
Oligomycin
ADP + Pi
PHOSPHORYLATION MECHANISM
m-Cl-CCP (uncoupler)
Isocitrate
α-ketoglutarate
MALATE
Pyruvate
NAD
FP
Amytal
Co Q
b
c
a
O2
FP
SUCCINATE
TTFA
AA
Cyanide
Fe(CN)6-3
TMPD
Ascorbate

Fig. II-1. Illustration of oxidative phosphorylation in mitochondria including sites of action of uncoupler and inhibitors of electron transfer and phosphorylation. Abbreviations: FP=flavoprotein; Co Q=coenzyme Q; b, c, and a = b-, c-, and a-type cytochromes; TTFA=thenoyltrifluoroacetone; AA=antimycin A; TMPD= tetramethylphenylene diamine; m-Cl-CCP=carbonylcyanide m-chlorophenylhydrazone; Pi=inorganic phosphate.

hydroxyquinoline N-oxide), and at the cytochrome oxidase region (e.g., cyanide, azide) (see Lehninger, 1965; Slater, 1966).

In order to locate the site of herbicide phytotoxicity in the respiratory chain, we employed techniques of feeding and withdrawing electrons at c-type cytochromes. In the presence of cyanide and ferricyanide, electrons from substrates can be directed to the reduction of ferricyanide (Estabrook, 1961; Lee et al., 1967). In the presence of antimycin A and ascorbate + tetramethylphenylene diamine (TMPD, used as the electron mediator between ascorbate and c-type cytochromes), the section of the respiratory chain between c-type cytochromes and oxygen can be studied (Sanadi and Jacobs, 1967). When one compares the patterns of oxygen uptake and ferricyanide reduction with succinate and an NAD-linked substrate, one can examine a possible site of herbicidal action between substrate and coenzyme Q and/or between coenzyme Q and c-type cytochromes. Furthermore, since electron carriers of the respiratory chain absorb light at specific wavelengths when they are reduced, difference spectra between treatments can provide further insight into the possible site of inhibition by a herbicide (see Chance, 1952; Chance and Williams, 1955b). Whenever appropriate, these analytical techniques were applied in our study.

The respiratory electron transfer chain is coupled to the phosphorylation mechanism at three sites; i.e., at the NAD-linked flavoprotein region, at the b- and c-type cytochrome region, and at the a-type cytochrome to cytochrome oxidase region (Chance and Williams, 1956; Lehninger, 1965; Slater, 1966).

The exact sites and nature of coupling of electron transfer to phosphorylation and also the exact mechanism of phosphorylation are still unknown and have been the subjects of intense investigations in many laboratories for many years (see reviews by Chance and Williams, 1956; Green, 1974; Greville, 1969; Slater, 1966). It has, however, been known that a class of compounds called uncouplers (phenolic compounds such as DNP and carbonylcyanide phenylhydrazone derivatives such as m-Cl-CCP) act to uncouple phosphorylation from electron transfer (Heytler, 1963; Heytler and Prichard, 1962; Ikuma and Bonner, 1967b; Lardy and Wellman, 1953; Lehninger, 1965; Slater, 1963, 1966). Uncouplers release the tight coupling of phosphorylation to electron transfer, stimulate electron transfer, decrease ATP formation, and increase the activity of ATP hydrolysis (see Slater, 1963). The phosphorylation mechanism is inhibited by a classical compound, oligomycin (Huijing and Slater, 1961; Lardy and McMurray, 1959; Lardy et al., 1958; Slater, 1963). Oligomycin inhibits phosphorylating rates of oxygen consumption by mitochondria, but the inhibition is reversed by an uncoupler (Huijing and Slater, 1961; Ikuma and Bonner, 1967; Lardy et al., 1958; Slater, 1963). This fact can be used to analyze whether a given herbicide acts to inhibit or uncouple phosphorylation.

It should be mentioned here that, in closer examinations, higher plant mitochondria exhibit many respiratory activities different from those of mammalian mitochondria (see Ikuma, 1972; Meeuse, 1975). For example, a cyanide insensitive pathway has been known to operate in many preparations of higher plant mitochondria, and the number and the light absorption peaks of electron carriers are not necessarily the same between the two types of mitochondria. These and other differences are found not to be important considerations in the analyses of phytotoxic action of herbicides. Thus, in the above description of the respiratory properties of higher plant mitochondria, we did not emphasize the differences. Nevertheless, because of differences recognized, it is highly important to use higher plant mitochondria, rather than rat liver or beef heart mitochondria which have been frequently employed in mitochondrial studies, in the analyses of herbicidal phytotoxicity.

A possible mode of action of herbicides on respiratory functions of isolated mitochondria can be seen in one or more of the following three ways: (1) inhibition of electron transfer, (2) inhibition of phosphorylation, and (3) uncoupling of phosphorylation from electron transfer (see Moreland, 1967). In the herbicide literature, these three aspects have been examined with a heavy emphasis on uncoupling of oxidative phosphorylation. For example, practically all phenolic herbicides examined, some dinitroaniline herbicides (nitralin and oryzalin), and substituted ureas (fluometuron and MBPU) are reported to act as uncouplers (McDaniel and Frans, 1969; Moreland, 1967; Moreland and Blackmon, 1970; Moreland and Boots, 1971; Moreland et al., 1974; Paton and Smith, 1967; Wang et al., 1974). These herbicides usually inhibit electron transfer at concentrations higher than those for uncoupling. A dinitroaniline derivative (trifluralin) and diphenylether herbicides are shown to act as electron transport inhibitors (Moreland et al., 1970; Wang et al., 1974). With a few herbicides, a possible interference of phosphorylation has been implicated (McDaniel and Frans, 1969; Wang et al., 1974). Triazines and phenoxys appear to act either weakly or at very high concentrations (Lotlikar et al., 1968; Thompson et al., 1974).

We analyzed possible modes of action of herbicides in three probable ways described above, using mitochondria isolated from dark grown mung bean hypocotyls and from corn roots. Succinate and malate were used as the major substrate in our standard screening, while other substrates were used as needed. In the case of inhibition of electron transfer, we made attempts to localize the site of inhibition in the respiratory chain. When the phosphorylation mechanism was suspected to be inhibited, an attempt was made to examine the effect of herbicide on the activity of ATPase.

MATERIALS AND METHODS

Isolation of Mung Bean Hypocotyl Mitochondria. Mitochondria was isolated by the method of Ikuma (1970) from the hypocotyls of 4-5 day old mung bean (Phaseolus aureus L.) seedlings grown in 8" x 12" x 2½" polystyrene planting trays in the dark. The hypocotyls were separated from roots and cotyledons + epicotyls by means of scissors, washed at least twice with distilled water to minimize any contamination, and, if necessary, stored in the cold room (0°-4° C) overnight in cold distilled water. The procedure to follow was carried out in the cold room, in an ice bath, in a Sorvall RC-2 or RC-5 refrigerated centrifuge set at 1° C, or with an ice-cold medium.

About 1.5 kg of hypocotyls were washed twice with distilled water, then cut into sections of about 2 cm, and placed in the grinding medium (0.5 M mannitol, 10 mM potassium phosphate, 1 mM EDTA, 0.2% bovine serum albumin, and 5 mM cysteine; cysteine was added immediately before grinding, and the starting pH was adjusted to about 8 with 10 N KOH). The cut sections and medium (volume ratio of 1 : 1) were placed in a porcelain mortar (capacity 750 ml) and ground by hand with a pestle until about two-thirds of the sections appear to have been crushed. During these steps, the pH of the tissue-medium mixture was maintained at 7.2 to 7.4 by dropwise additions of 10 N KOH. The resultant brei was strained through at least eight layers of cheesecloth. The residue was re-extracted with about one-third volume of the grinding medium. The combined filtrate was subjected to differential centrifugation.

A crude mitochondrial pellet was collected between 500 x *g* (10 minutes) and 12,000 x *g* (15 minutes), and is homogeneously suspended in about 30 ml of wash medium (0.5 M mannitol, 10 mM potassium phosphate buffer, pH 7.4, and 0.2% bovine serum albumin). The suspension was subjected to one spin each of low (500 x *g*, 15 minutes) and high speed (6,000 x *g*, 15 minutes). The pellet from the high speed spir was homogeneously suspended in about 30 ml of wash medium, and centrifuged at 5,500 x *g*.

The final pellet contained the washed mitochondria (abbreviated in figures as Mw) which was suspended in a small volume of wash medium yielding about 40 mg protein/ml.

Utmost care was given to the tissue disruption step, the pH during grinding, and the rigorous exclusion of contaminating particles during centrifugation. Unless the above care was not well observed, the isolated mitochondria lost the activity within a few hours.

Isolation of Corn Root Mitochondria. Corn seeds (Zea mays L. Michigan Hybrid 200) were soaked in a 5% (v/v) commercial Clorox solution for 15 minutes, rinsed thoroughly with tap water, imbibed in tap water overnight, germinated on perforated aluminum sheets in 8" x 12" x 2½" planting trays for 2-3 days in the dark, and grown for additional 2-3 days in the green house. Roots were then harvested and rinsed several changes of cold distilled water to remove foreign materials.

Rinsed roots (about 500 g) were placed in a porcelain mortar (750 ml capacity) with the grinding medium, cut into small pieces with a pair of scissors, and ground by hand with a pestle until most root pieces appeared to have been crushed. During grinding, the pH was carefully monitored to 7.2 to 7.8 by dropwise additions of 5 N KOH. The resultant brei was strained through at least eight layers of cheesecloth. The filtrate was subjected to differential centrifugation in the same manner as for mung bean hypocotyl mitochondria.

The isolation of corn root mitochondria posed more problems than we originally anticipated. Problems were mainly caused by low yield of mitochondria, hard cell wall of corn roots, and apparent fragility of isolated mitochondria. The grinding step was the most important step to alleviate the subsequent problems. Utmost care was given not to overgrind nor undergrind the root pieces. The pH change during grinding of corn roots was much smaller than that of mung bean hypocotyls. The maintenance of the pH in the vicinity of 7.4 was found to be very important during the grinding process. After many initial failures, we learned that sorbitol at 0.7 M was a better osmoticum for corn root mitochondria than 0.5 M mannitol or sucrose. We thus replaced 0.5 M mannitol with 0.7 M sorbitol in the grinding and washmedia for the isolation of corn root mitochondria; other components were the same as those for mung bean mitochondria. Re-extraction of tissue residues after straining was frequently found to decrease the quality of mitochondria; this step was thus omitted in the preparation of corn root mitochondria. Since the first low speed spin (500 x g, 10 minutes) did not yield appreciable pellet, we often omitted this spin in later preparations. It should be mentioned that we made several attempts to purify high quality mitochondria by means of discontinuous sucrose or sorbitol density gradient centrifugation (Douce et al., 1972); these attempts resulted in no clear improvement on the quality of mitochondria, and differential centrifugation was used as the standard procedure for isolation of corn root mitochondria.

In contrast to mung bean mitochondria which consistently showed tight coupling, corn root mitochondria frequently showed poor respiratory and phosphorylative activities. In addition, corn root mitochondria were apparently deficient in thiamine pyrophosphate upon isolation; this cofactor was thus routinely added to the reaction medium. Only the mitochondrial preparations whose respiratory control and phosphorylative activity were comparable to mung bean hypocotyl mitochondria were used in our analyses.

Preparation of Crude Malate Dehydrogenase from Mitochondria. Malate dehydrogenase was extracted from isolated mitochondria in a crude form. A mitochondrial suspension was first frozen and thawed, and a given volume of cold 5 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA and 1 mM β-mercaptoethanol was added. The mixture was then stirred for 10 minutes at 0° C and centrifuged at 27,000 x g for 15 minutes. The pellet was re-extracted with one half volume of the extraction medium and the mixture was centrifuged as before. The combined supernatant was used as the enzyme source.

The volume ratio of initial mitochondria to phosphate buffer extraction medium was about 5 mg protein/ml medium. The final combined supernatant contained 95% or more of total malate dehydrogenase activity detectable in the original mitochondria and also very little activities of other enzymes (Bowman et al., 1975).

Equipment, Assay Media, and Chemicals. Oxygen consumption by mitochondria was measured at 25° C in a stirred Lucite cuvette of 3 ml capacity with a YSI oxygen electrode (No. 4004, Yellow Springs Instrument Co., Yellow Springs, Ohio) which was connected to a home-made circuit box (see Estabrook, 1967), and a Heath-Schlumberger recorder Model SR-255-B.

Spectrophotometric assays were carried out using any one of 4 spectrophotometers available: Zeiss spectrophotometers PMQ II and PMQ III (the latter was made to a recording spectrophotometer by connecting to a Heath-Schlumberger recorder Model SR-255-B), and Cary recording spectrophotometers Models 13 and 17. When a sample of high turbidity was employed such as mitochondria, a Cary spectrophotometer Model 13 with a light scattering attachment was used.

The standard reaction media consisted of 0.5 M mannitol or 0.7 M sorbitol, 10 mM potassium phosphate buffer, pH 7.2, 10 mM KCl, and 5 mM $MgCl_2$. The mannitol medium was used for mung bean mitochondria and the sorbitol medium for corn root mitochondria. Immediately before the assay of corn root mitochondrial activity, 100 to 200 μM thiamine pyrophosphate was added to the reaction medium. Additions of other compounds are noted in the RESULTS and made in small volumes of 5 to 60 ul.

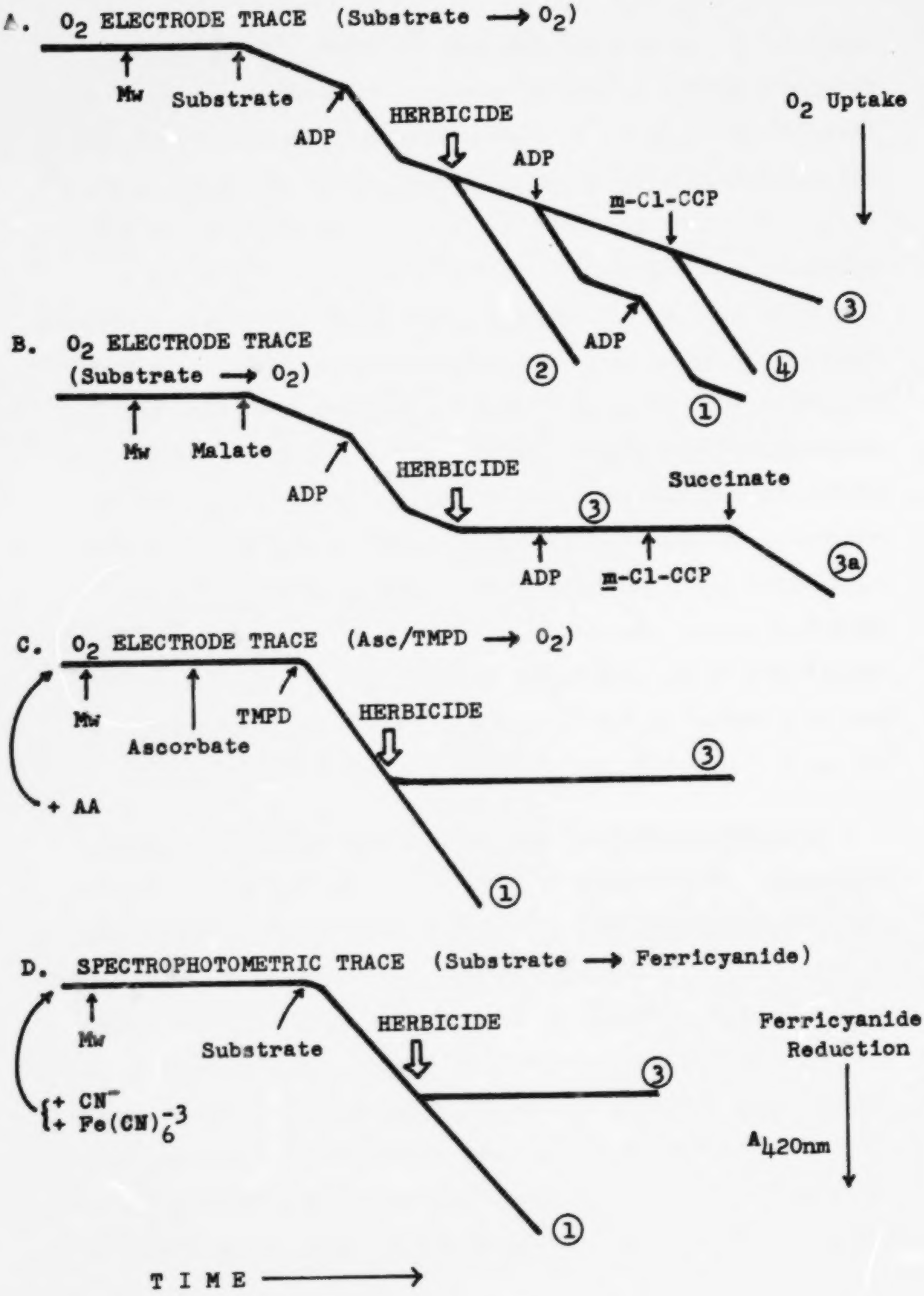
Since many herbicides tested were relatively insoluble in water, stock solutions were made with dimethylsulfoxide (DMSO). The concentration of DMSO in the reaction cuvette was less than 1% at which DMSO did not exert any significant effect on mitochondrial activity (cf. McDaniel and Frans, 1969; Wang et al., 1974). At this low concentration of DMSO, herbicides frequently precipitated, when their concentrations exceeded above 0.1 mM. The stock solutions of oligomycin, antimycin A, and m-Cl-CCP were prepared in absolute ethanol. Whenever DMSO or ethanol dissolved chemicals were tested, control runs were made with the same concentrations of DMSO and ethanol.

All chemicals were of analytical reagent grade and the best commercially available grade. Bovine albumin fraction V powder, used for mitochondrial isolatio, and crystalline BSA, used for standard curves of proteins, were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. ATP, ADP, thiamine pyrophosphate, and carbonylcyanide m-chlorophenyl hydrazone (m-Cl-CCP) were purchased from Calbiochem. NAD, NADH, oligomycin, and antimycin A were obtained from Sigma Chemical Co. Lactic dehydrogenase, pyruvate kinase, and adenylate kinase were purchased from Boehringer-Mannheim Corp. The concentrations of NADH and ADP were determined optically at 340 nm and 260 nm on the basis of extinction coefficients of 6.22 and 15.4 $mM^{-1}.cm^{-1}$, respectively.

Protein was determined by a biuret procedure (Gornal et al., 1949) after solubilization of mitochondria with desoxycholate, with crystalline bovine albumin as the standard and the wash medium as the blank. With mitochondrial extracts, protein content was measured with the more sensitive method of Lowry et al. (1951).

Standard Screening Method with an Oxygen Electrode. The major screening method of herbicidal phytotoxicity used in this study is illustrated in Fig. II-2A. Herbicides are

Fig. II-2. Expected recorder traces showing possible modes of action of herbicides. Encircled numbers on each trace indicate, as follows: ①, no effect of herbicide or the same as control; ②, herbicide acting as an uncoupler; ③, herbicide acting as an inhibitor of electron transfer; (3a), a modified form of inhibition of electron transfer; and ④, herbicide acting as an inhibitor of phosphorylation. A, the standard screening procedure with an oxygen electrode using succinate or malate as substrate. B, complete inhibition of malate oxidation by herbicide, which is partially reversed by succinate addition. C, cytochrome oxidase assay with and oxygen electrode using ascorbate as the electron donor and TMPD as the electron mediator. D, spectrophotometric trace of ferricyanide reduction at 420 nm.


A. O_2 ELECTRODE TRACE (Substrate → O_2)
Mw
Substrate
ADP
HERBICIDE
ADP
$\underline{m}$-Cl-CCP
ADP
O_2 Uptake
③
②
①
④
B. O_2 ELECTRODE TRACE (Substrate → O_2)
Mw
Malate
ADP
HERBICIDE
③
Succinate
ADP
$\underline{m}$-Cl-CCP
3a
C. O_2 ELECTRODE TRACE (Asc/TMPD → O_2)
Mw
Ascorbate
TMPD
HERBICIDE
③
①
+ AA
D. SPECTROPHOTOMETRIC TRACE (Substrate → Ferricyanide)
Mw
Substrate
HERBICIDE
③
①
+ CN^-
+ $Fe(CN)_6^{-3}$
Ferricyanide Reduction
A_{420nm}
T I M E →

expected (1) to inhibit electron transfer, (2) to inhibit phosphorylation, or (3) to uncouple oxidative phosphorylation (see INTRODUCTION). If a given herbicide does not affect the mitochondrial activity at all, there is no immediate action after herbicidal addition, and subsequent additions of ADP stimulate respiration as the first ADP (line ①). If a herbicide acts as an uncoupler, the respiratory rate increases immediately after herbicide addition (line ②). If a herbicide acts as an inhibitor of electron transfer, the respiratory rate usually change little immediately after the herbicide addition, but the subsequent additions of ADP and an uncoupler (m-Cl-CCP in this figure) do not stimulate the rate (line ③). If a herbicide acts as an inhibitor of phosphorylation, the respiratory rate does not change appreciably immediately after additions of herbicide and the second ADP, but is expected to increase drastically after an addition of m-Cl-CCP (line ④).

Analysis of Site of Herbicidal Inhibition of Electron Transport. If a herbicide inhibits at the NAD-linked flavoprotein region, but not at the succinate-linked flavoprotein region, or if a herbicide inhibits the oxidation of NAD-linked substrate more effectively than that of succinate as in the case of amytal (Ikuma and Bonner, 1967c), the expected trace of an oxygen electrode is as illustrated in Fig. II-2B. Here herbicide inhibition of electron transport from malate, an NAD-linked substrate, is complete even after additions of ADP and m-Cl-CCP, but the inhibition is released by succinate.

Although not shown in Fig. II-2, if inhibition of electron transport should occur at the flavoprotein level of succinate oxidation, succinate oxidation would be completely inhibited after herbicide addition, but the inhibition would be reversed by an addition of a NAD-linked substrate. Complete or differential inhibition of oxidation of succinate or NAD-linked substrates indicates that the site of action is near to the substrate before the branching point of the respiratory chain (see Fig. II-1).

Possible herbicidal inhibition of cytochrome oxidase can be examined with an oxygen electrode using ascorbate as the electron donor and TMPD as the electron mediator in antimycin A-inhibited mitochondria (see Fig. II-1). Such inhibition of cytochrome oxidase by a herbicide is illustrated in Fig. II-2C.

Possible inhibition of electron transport between substrate and _c_-type cytochromes can be examined spectrophotometrically using terminally inhibited mitochondria and following ferricyanide reduction at 420 nm (line ③ in Fig. II-2D). If the inhibition occurs for the oxidation of both succinate and malate, the site of inhibition must be in the common electron transport chain. If the inhibition is restricted to the oxidation of either substrate, the site of inhibition must be in the substrate side of the branch of the respiratory chain.

In order to supplement and also pin-point the site of inhibition, difference spectra were taken using a Cary Model 13 recording spectrophotometer with a light scattering attachment (Chance and Williams, 1955).

In the case of diallate, inhibition of malate oxidation was complete in a manner as shown in Fig. II-2B. In addition, the study with difference spectra showed that the site of herbicidal action was in the substrate side of spectrophotometrically detectable flavoprotein. We thus examined the effect of this herbicide on the activity of malate dehydrogenase by means of NADH oxidation with a recording spectrophotometer (Bowman and Ikuma, 1975). The reaction medium contained 50 mM potassium phosphate buffer at pH 8.0, 0.15 mM NADH, about 0.2 μg enzyme protein, and 0.2 mM oxaloacetate. The addition of oxaloacetate initiated the reaction. It should be mentioned that the activity of NADH oxidase was negligible at this low concentration of the crude mitochondrial extract used in the assay.

ATPase Assay. If a herbicide is suspected, from the oxygen electrode studies, to act as an inhibitor of phosphorylation, it is of interest to see whether the herbicide acts in a manner similar to oligomycin, which inhibits ATPase as well as phosphorylation. An addition of 1 mM ATP to mitochondria in the standard reaction medium initiated the reaction. After 5 minutes at 25° C, the reaction was stopped by an addition of perchloric acid to give a final concentration of 3.3%. The acid mixture was centrifuged at 12,000 x g for 10 minutes to remove precipitated proteins. A measured volume of the clear supernatant was pipetted into a test tube and the pH was raised to about 6 with 3 N KOH. During this pH adjustment step, perchloric acid was precipitated as potassium perchlorate and the precipitate removed by centrifugation at 12,000 x *g* for 10 minutes. The resultant clear supernatant was used for ADP determinations with a recording spectrophotometer at 340 nm using coupled reaction of lactate dehydrogenase, pyruvate kinase, and adenylate kinase (Williamson and Corkey, 1969). During the preparation of perchloric acid extract of mitochondria, the volumes of acid and other additives and also of supernatants were recorded to facilitate final calculation of ATPase activity. It should be mentioned that a more commonly employed method of phosphate-colorimetric assay was not used in our study, because the colorimetric method was not as sensitive as the ADP determination.

RESULTS

General Survey of the Effects of Seventeen Herbicides. The survey of phytotoxicity of 17 herbicides on the respiratory activities of isolated mitochondria was performed with the standard screening procedure illustrated in Fig. 2A. With mung bean hypocotyl mitochondria complete survey was carried out with both succinate and malate as substrate, while with corn root mitochondria only malate was used as substrate. The results with two types of mitochondria were practically identical. Slight differences observed were mainly in the values of half-maximal inhibition and/or in the degrees of maximal inhibition. In view of close similarity of the two types of mitochondria, the results with mung bean mitochondria are primarily given in this report.

Of the seventeen herbicides tested, seven herbicides gave very little or no effect at the saturating or supersaturating concentrations (1-10 mM) in both mung bean and corn root mitochondria. They were amitrole, atrazine, cacodylate, diquat, DSMA, karbutylate, and simazine. Any effect observed with these herbicides was less than 25% (in most cases 0%) inhibition of electron transport at a 5-10 mM range.

Another group of seven herbicides affected the activity of mung bean mitochondria at or above the limit of their solubility (0.1-10 mM) in our standard screening procedure (Table II-1). Bromacil, propham, chlorpropham, and 2,4-D inhibited the second state 3 rate of succinate oxidation only partially, while they inhibited more than 75% of malate state 3 rates. These herbicides thus appear to inhibit NAD-linked flavoprotein more markedly than succinate-linked flavoprotein. The two urea type herbicides, monuron and diuron, acted to uncouple oxidative phosphorylation, particularly with succinate as substrate. Monuron was more effective than diuron. 2,4,5-T inhibited both malate and succinate oxidation maximally, but the half-maximal inhibition of electron transport was attained at a lower concentration for malate oxidation than for succinate oxidation. The degrees of maximal inhibition and the values for 50% inhibition of electron transport given in Table II-1 for mung bean mitochondria were repeated in a practically identical manner with corn root mitochondria. In view of the high titer values, particularly in comparison with the

TABLE II-1. Effects of weakly active herbicides on respiratory activities of mung bean mitochondria.

The standard screening procedure was used with 10 mM succinate or 30 mM malate and 150 μM ADP. The I_{50} value refers to the concentration of a herbicide which inhibits 50% of the second state 3 rate.

Herbicide	Substrate	I_{50} (mM)	Remarks
Bromacil	Succinate	....	Max. inhibition 37% at 10 mM
	Malate	1.6	80% inhibition at 10 mM
Chlorpropham	Succinate	....	Max. inhibition 40% at 10 mM
	Malate	0.7	80% inhibition at 10 mM
Propham	Succinate	....	Max. inhibition 40% at 10 mM
	Malate	0.8	85% inhibition at 10 mM
Diuron	Succinate	....	Slight uncoupling at 0.3-1 mM
	Malate	0.2	90% inhibition at 3 mM
Monuron	Succinate	....	Max. uncoupling at 0.3-1 mM
	Malate	....	Uncoupling at 1 mM
2,4-D	Succinate	....	Max. inhibition 50% at 3 mM
	Malate	0.6	Max. inhibition 75% at 3 mM
2,4,5-T	Succinate	0.9	Max. inhibition 95% at 3 mM
	Malate	0.2	Max. inhibition 95% at 3 mM

values for inhibition of photosynthetic electron transport (see next Chapter), their primary sites of action can be considered not in the mitochondrial activity. Furthermore, any effects at millimolar concentrations or super-saturating concentrations may result from possible contaminants in the chemicals. We have not, therefore, pursued the modes of action of these herbicides further in our study.

The remaining three herbicides, DNOC, trifluralin, and diallate, affected mitochondrial activities at a micromolar level. In the following sections, therefore, the action of these herbicides is described individually.

Action of DNOC. Phenolic herbicides have been shown to uncouple oxidative phosphorylation of isolated mitochondria by many workers (Ashton and Crafts, 1973; Corbett, 1974; Herbicide Handbook, 1974; Moreland, 1967; Moreland and Blackmon, 1970; Moreland et al., 1974; Paton and Smith, 1967; Parker, 1965). When DNOC is added during the second state 4, the respiratory rate increases as illustrated in line ② of Fig. II-2A with either succinate or malate as substrate. The titration curve for succinate oxidation (Fig. II-3) is identical with the curve for malate oxidation (not shown) with the maximal uncoupling occurring at 30 µM in mung bean mitochondria. With corn root mitochondria, the maximal uncoupling took place at 20 µM. Similar to other known uncouplers, such as DNP and m-Cl-CCP, high concentrations inhibit electron transfer (Ikuma and Bonner, 1967b). A further similarity of DNOC to classical uncouplers is seen in Fig. II-4, where this herbicide acts to

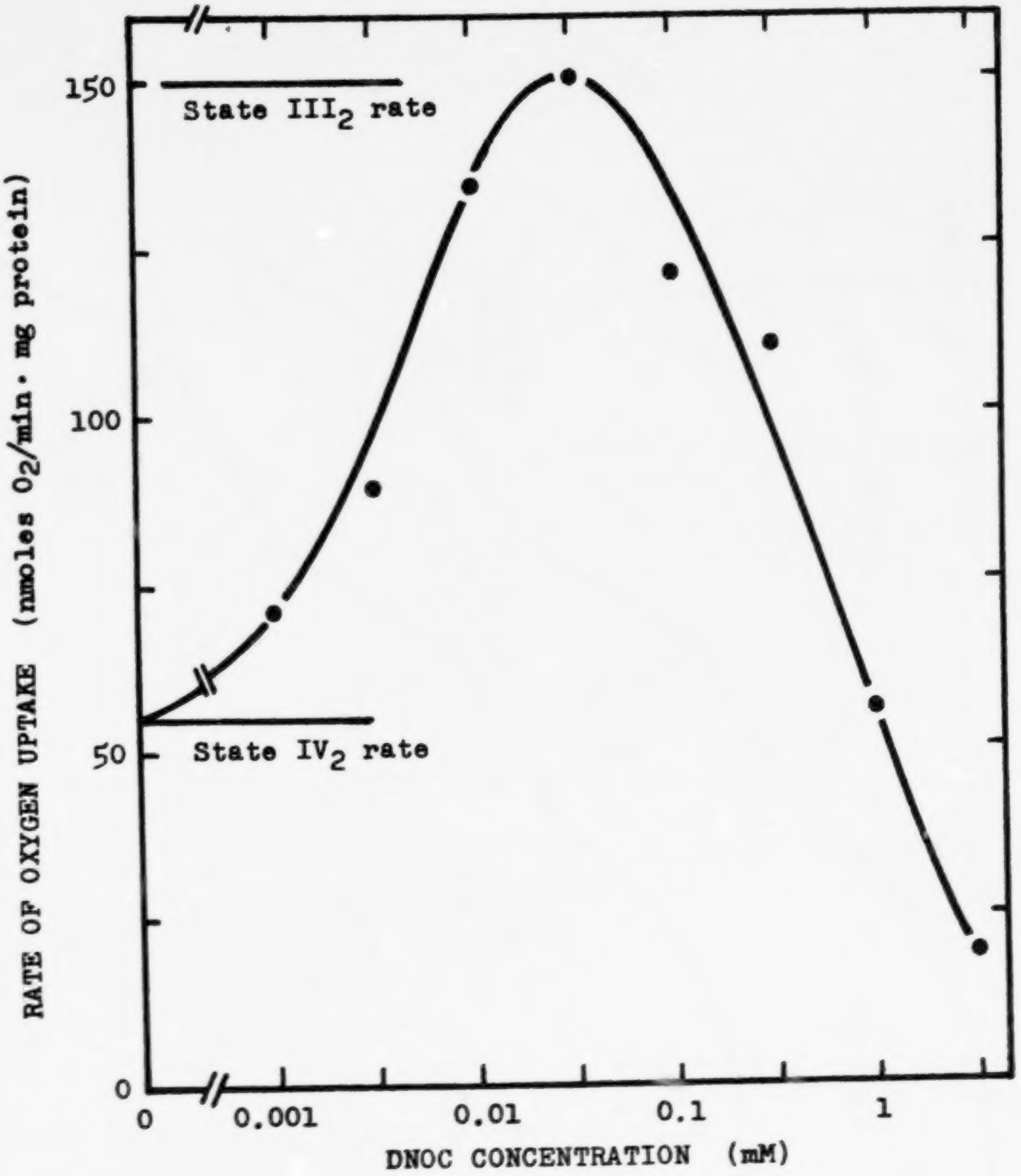


Fig. II-3. Titration of DNOC effect on succinate oxidation in mung bean mitochondria.

The release of coupling was examined with the standard screening procedure of Fig. II-2A. Concentrations used were: mitochondria=1.35 mg protein, succinate as substrate=10 mM, ADP for the first state 3-4 transition= 170 µM.

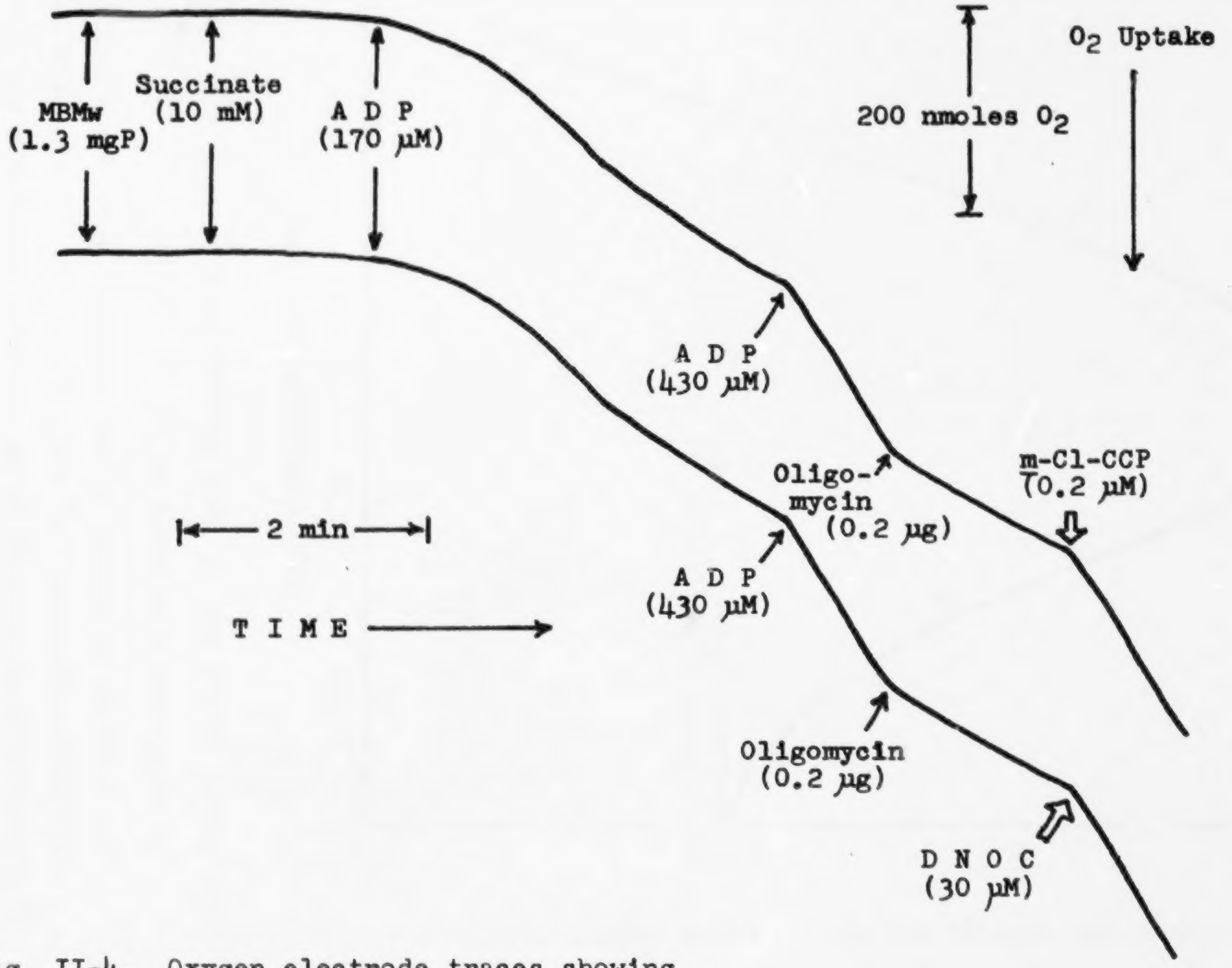


Fig. II-4. Oxygen electrode traces showing reversal of oligomycin inhibition by DNOC and m-Cl-CCP.

release oligomycin inhibition of state 3 rate.

DNOC is, however, different from m-Cl-CCP in that DNOC does not bind with mitochondria stoichiometrically. Although the results are not shown here, DNOC-pretreated mitochondria recovered full coupling of phosphorylation to electron transfer after three washings. On the other hand, m-Cl-CCP has been shown to bind with mitochondria stoichiometrically in a manner similar to antimycin A and oligomycin (Slater, 1963).

Action of Trifluralin. Figure II-5 illustrates titration curves of trifluralin inhibition of both succinate and malate oxidation with mung bean mitochondria. The results indicate that (a) two curves parallel each other well, (b) maximal inhibition approaches the state 4 level, and (c) the half-maximal inhibition is obtained at about 30 μM, assuming the full inhibition to be the state 4 levels for the oxidation of both substrates. In another experiment, titration curves were examined on succinate oxidation with two different concentrations of mung bean mitochondria (Fig. II-6). In this experiment, maximal inhibition reached the state 4 level and the titration curves for two mitochondrial concentration was practically identical with a half-maximal inhibition to be about 20 μM. In the two series of experiments illustrated and in several other experiments, trifluralin caused practically no inhibitory effect on the state 4 respiration. In addition, the half-maximal inhibition value was about 20 μM in several other experiments including the ferricyanide reduction assay

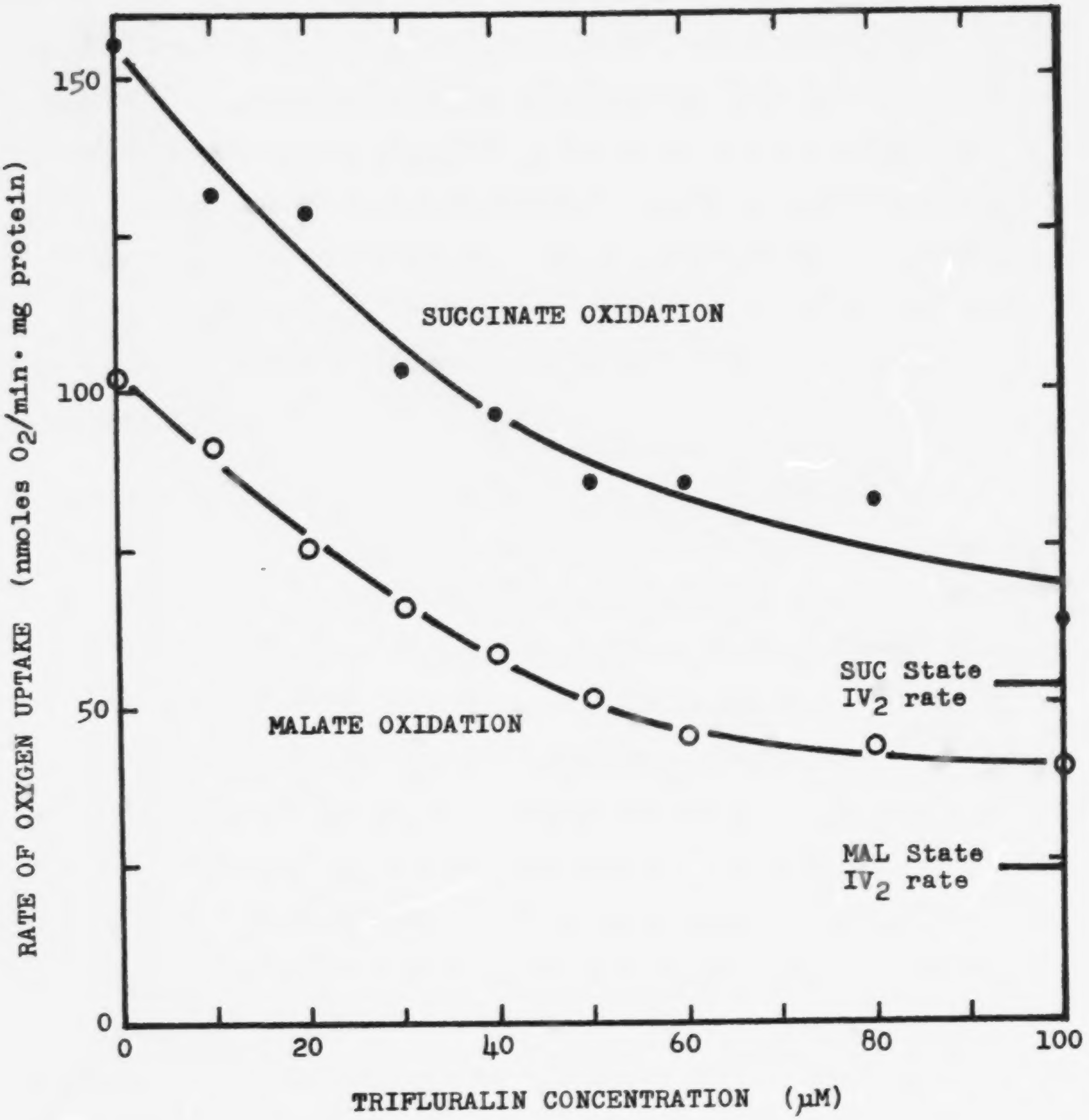


Fig. II-5. Titration of trifluralin inhibition of state 3 rates of succinate and malate oxidation in mung bean mitochondria. Concentrations used were: mitochondria=1.5 mg protein; Succinate=10 mM; Malate=30 mM; ADP=155 µM.

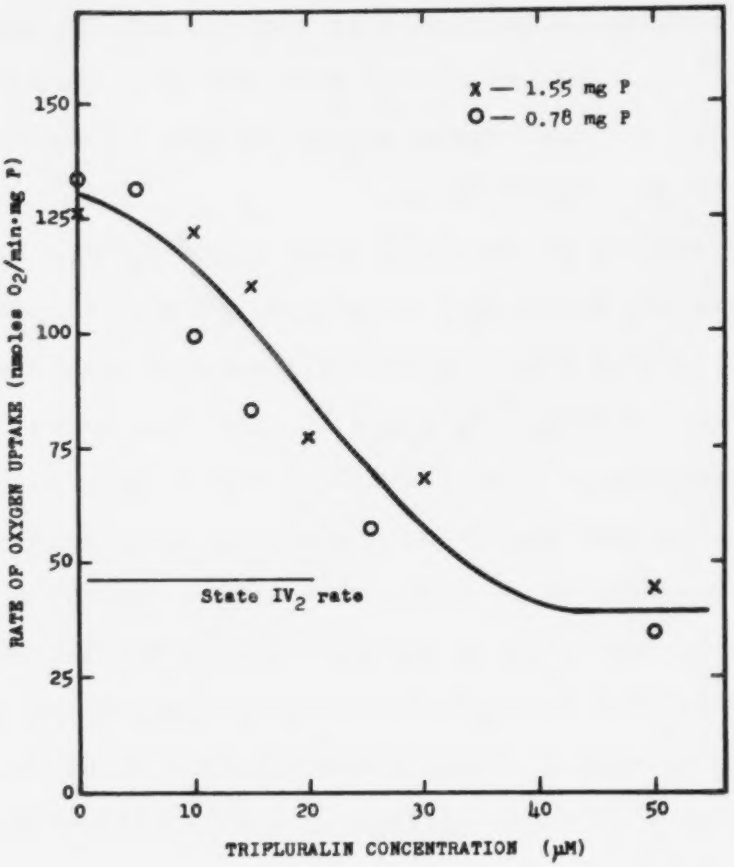


Fig. II-6. Titration of trifluralin effect on succinate oxidation with two concentrations of mung bean mitochondria.

Two concentrations of mitochondria used are given on the figure. 10 mM succinate and 170 µM ADP were used in the standard screening assay.

with either succinate or malate as substrate. The same titer value was obtained with corn root mitochondria on malate oxidation. It should be stated that 100 uM trifluralin did not at all affect the oxygen uptake pattern in the ascorbate/TMPD cytochrome oxidase assay.

The practically identical titer value for the oxidation of succinate and NAD-linked substrate and also no effect on cytochrome oxidase activity strongly suggest that the herbicide acts between the branching point of the respiratory chain and c-type cytochromes. This possiblity was further examined taking a difference spectrum of electron transfer carriers. In repeated attempts, however, we could not detect any difference in the spectrum between control and trifluralin treatment; all carriers remained reduced regardless of trifluralin treatment. High concentrations of mitochondria which were used in these attempts probably allowed mitochondria to become anaerobic quickly due mainly to incomplete inhibition by this herbicide (see Fig. II-5).

Another possible interpretation of the results thus far obtained is that trifluralin acts to inhibit the phosphorylation mechanism like oligomycin. We thus tested this possibility by additing ADP and m-Cl-CCP after trifluralin. Trifluralin concentration used in this experiment was 50 µM, in order to avoid precipitation of this herbicide. As illustrated in Fig. II-7, ADP was capable of stimulating respiration slightly with the concentration of trifluralin used. A subsequent addition of m-Cl-CCP could not stimulate respiration as in

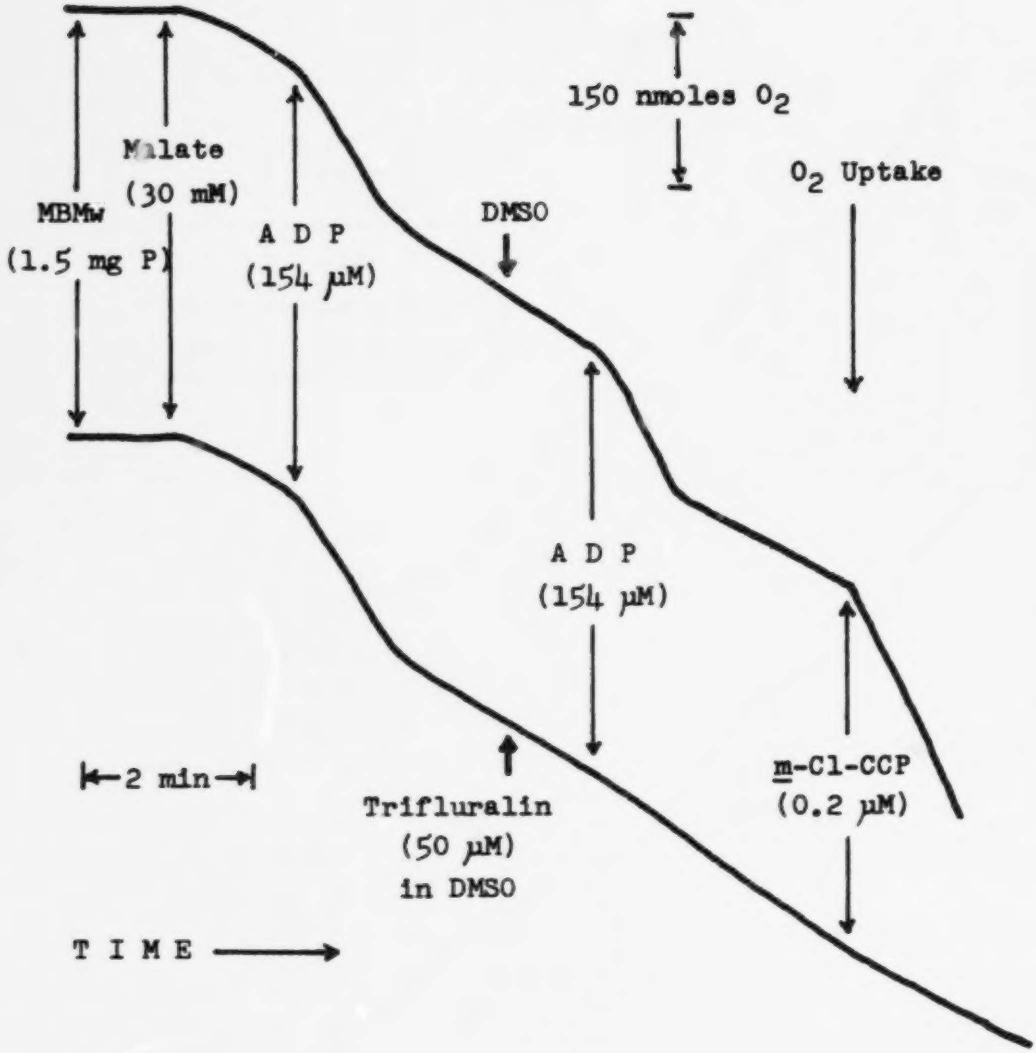
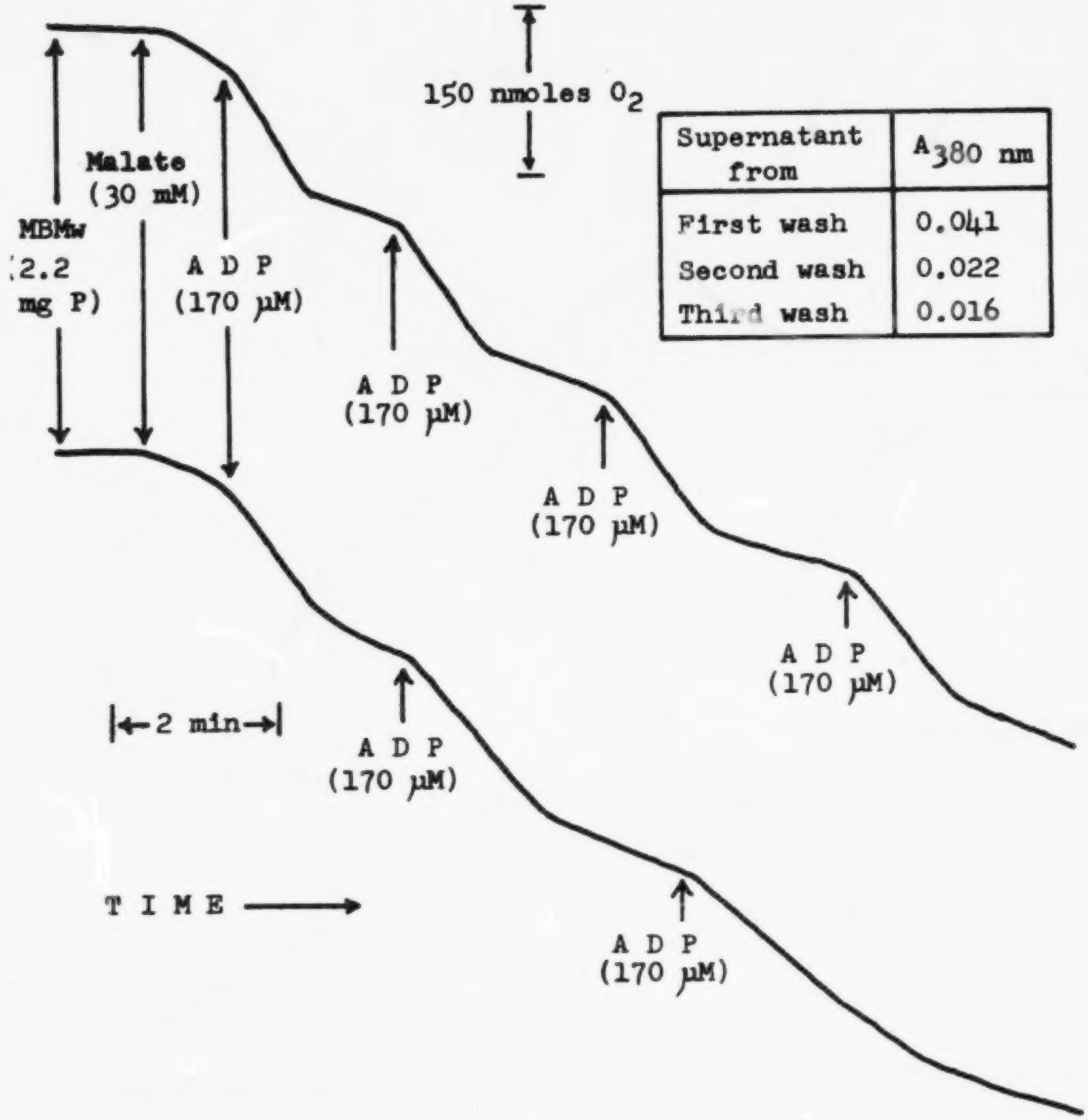


Fig. II-7. Oxygen electrode traces showing effect of m-Cl-CCP on trifluralin-inhibited mitochondria.

Upper trace = control. Lower trace = trifluralin treatment. MBMw = mung bean mitochondria. Numbers in parentheses indicate final concentrations of chemicals in the reaction medium.



Supernatant from	A_{380} nm
First wash	0.041
Second wash	0.022
Third wash	0.016

Fig. II-8. Examination of trifluralin binding to mung bean mitochondria.

Mitochondria (2.2 mg protein) were treated with 100 µM trifluralin at 4° C for 10 minutes, then washed three times with the wash medium of mitochondrial preparation, and tested for the oxidation of malate with an oxygen electrode. The resultant oxygen electrode traces are shown here; Upper trace=control, Lower trace=trifluralin pretreatment. Each wash step was carried out 3 ml wash medium and centrifugation; color appearance in the supernatants of three successive washes is given in the inset.

the control. In fact, the respiratory rate was reduced to the state 4 level after m-Cl-CCP addition. These results clearly indicate that trifluralin does not act like oligomycin.

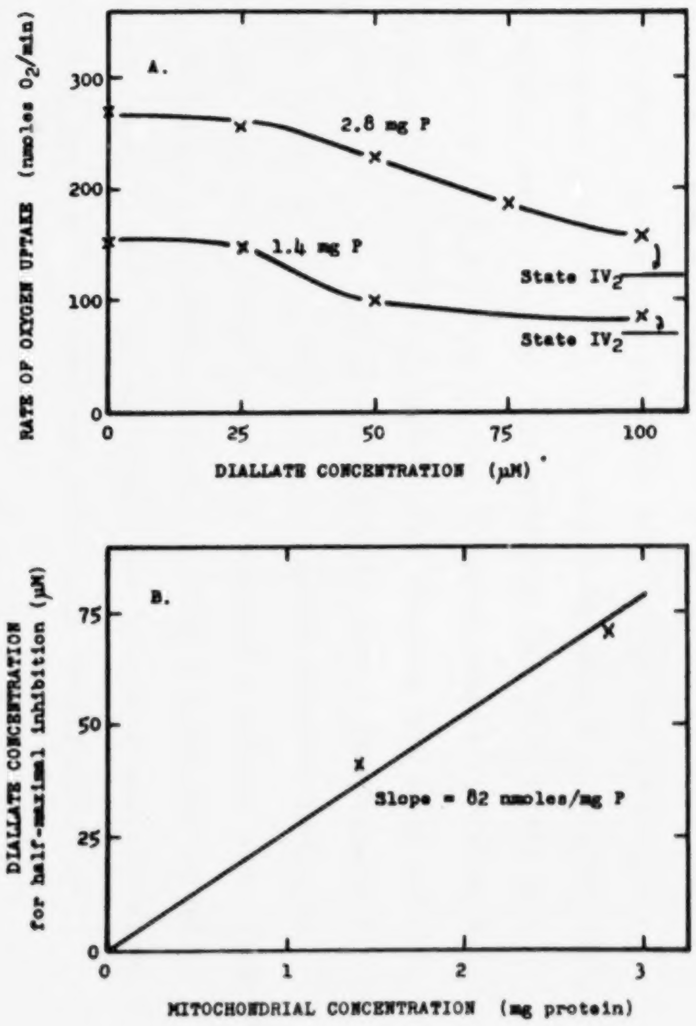
Trifluralin-treated mitochondria were stained yellow and in repeated washings the color faded slowly. In the experiment shown in Fig. II-8, mung bean mitochondria were treated with 100 uM trifluralin, then washed three times with the wash medium of mitochondrial preparation, and tested for malate oxidation with an oxygen electrode. The mitochondria were still stained lightly yellow. The results show that malate oxidation proceeds normally up through the first cycle of state 3-4 transition, then gradually slows down as state 3-4 transitions increase. This trace is similar to the trace with a low concentrations of trifluralin such as the one obtained with 10 μM concentration. Since trifluralin is relatively insoluble in water (solubility limit about 80-100 μM), it may have high lipid solubility and the bound trifluralin may be released slowly in accordance with its partition coefficient (see inset).

Action of Diallate. Figures II-9 and II-10 shows the titration of diallate to succinate and malate oxidation. In these experiments, diallate was added during the second state 4, and the effect was examined for inhibition of second state 3 rates. Figures II-9A and II-10A show that malate oxidation is nearly completely inhibited at 100 μM concentration of diallate, while inhibition of succinate oxidation is about 50% or close to the level of the non-phosphorylating state 4

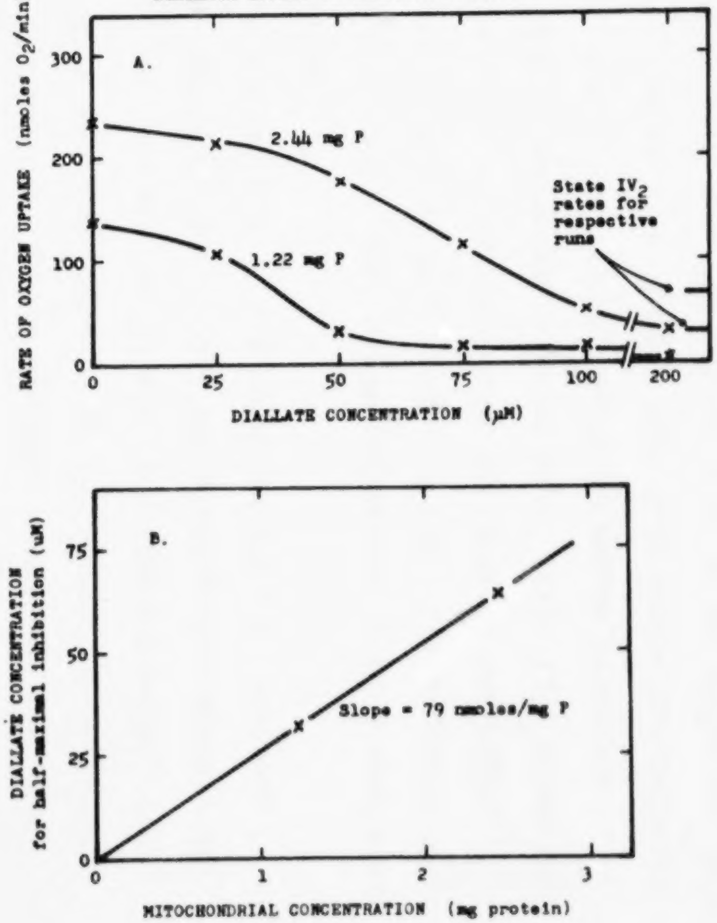
Fig. II-9. (Left figures) Titration of diallate effect on succinate oxidation with two different concentrations of mung bean mitochondria.
The standard screening method of Fig. II-2A was employed, and the effect of diallate on second state 3 rates were examined.

Fig. II-10. (Right figures) Titration of diallate effect on malate oxidation with two different concentrations of mung bean mitochondria.
The standard screening method, Fig. II-2A, was employed, and the effect of diallate on second state 3 rates were examined.

DIALLATE EFFECT ON SUCCINATE OXIDATION



DIALLATE EFFECT ON MALATE OXIDATION



rate, and titration curves are dependent on mitochondrial concentrations used. If one plots the diallate concentration which causes a half-maximal inhibition as a function of mitochondrial concentrations used, one obtains a linear relationship as shown in Figs. II-9B and II-10B. These results indicate that diallate binds stoichiometrically with mitochondria at about 80 nmoles per mg mitochondrial protein for both malate succinate state 3 oxidation. In another series of experiment, mitochondria were treated with diallate for 10 minutes and washed three times with 3 ml of the wash medium of mitochondrial preparation. These mitochondria were found not to oxidize malate at all and to oxidize succinate at a state 4 rate regardless of the presence of ADP (Fig. II-11). From these observations it can be concluded that the stoichiometric binding of diallate is irreversible. Diallate-inhibited mitochondria were *not* relieved of inhibition by sulfhydryl compounds such as mercaptoethanol and dithiothreotol, nor by bovine serum albumin. Diallate binding thus appears to be due to lipophilicity of the compound with the membrane phospholipid.

The observation that malate oxidation is more completely inhibited by diallate than succinate oxidation suggests that one of the sites of inhibition by diallate is the region between malate and *b*-type cytochromes. Table II-2 shows the effect of diallate on substrate-ferricyanide reductase activity. The results clearly show that malate ferricyanide reductase is more than 80% inhibited at 50 μM, while succinate-

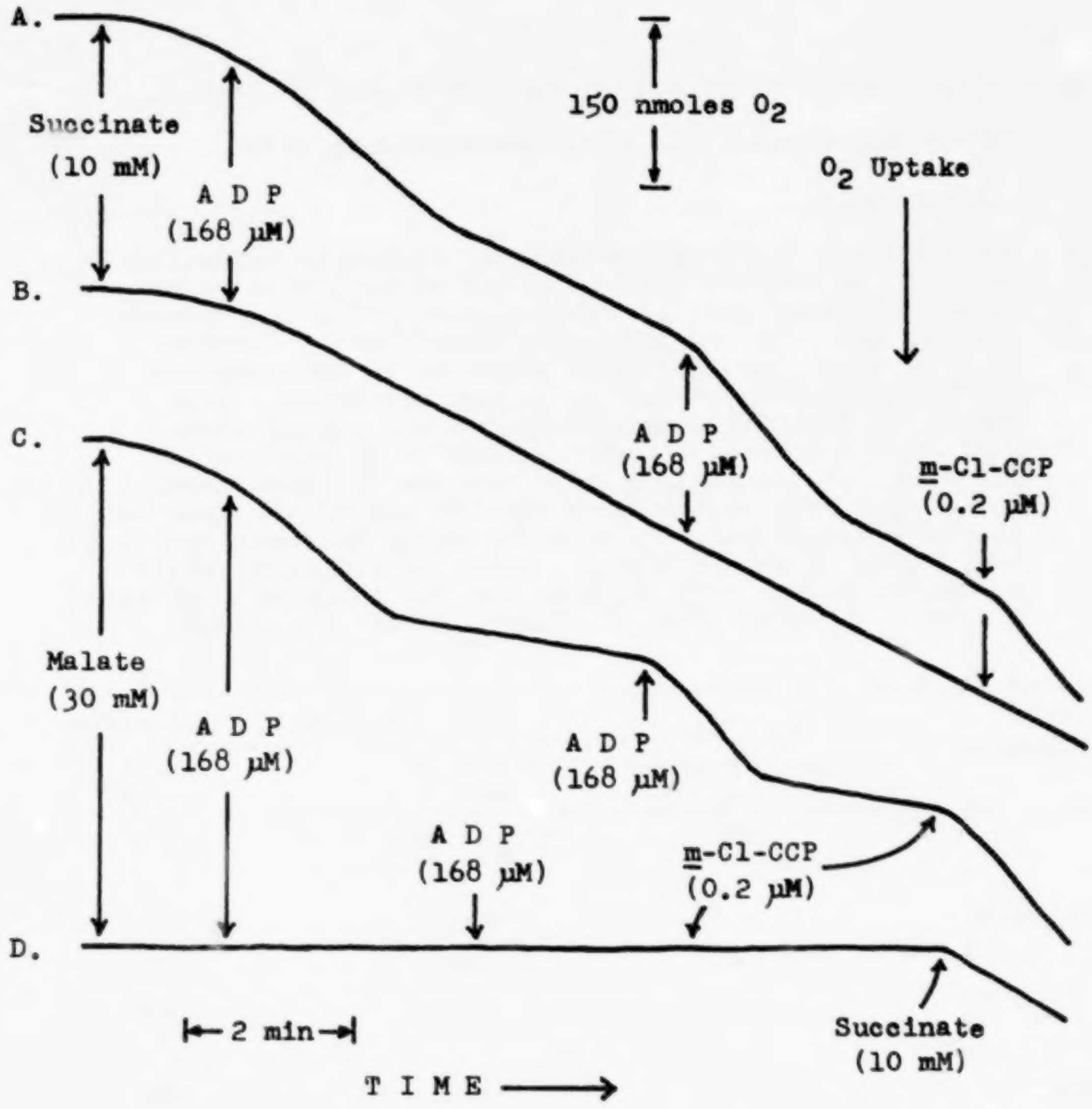


Fig. II-11. Examination of diallate binding to mung bean mitochondria.

Mitochondria (158 mg protein) were treated with 300 µM diallate at 4° C for 10 minutes, washed three times with the wash medium of mitochondrial preparation (3 ml each time), and tested for oxidation of succinate and malate as shown in the figure. A, Succinate oxidation by control mitochondria. B, Succinate oxidation by diallate-pretreated mitochondria. C, Malate oxidation by control mitochondria. D, Malate oxidation by diallate-pretreated mitochondria. Control mitochondria were not treated with diallate, but washed three times.

Table II-2. Diallate effect on succinate- and malate-ferricyanide reductase activities in mung bean mitochondria.

Mitochondria (1.56 mg protein for succinate oxidation and 1.77 mg protein for malate oxidation) were added to the standard reaction medium containing 1 mM ferricyanide and 1 mM KCN in a spectrophotometer cuvette ; then substrate and ADP were added as in the standard screening procedure with an oxygen electrode. With malate as substrate, spectrophotometer traces show state 3-4 transitions; with succinate as substrate, no state 3-4 transitions were observable (Estabrook, 1961). The assays with malate were carried out in the same manner as the standard oxygen electrode assay for examination of inhibition of second state 3 rates by diallate, while succinate assays were carried out for inhibition of state 4 rates. Succinate=10 mM; Malate=30 mM; ADP=77 µM.

Substrate	Diallate concentration	Rate of ferricyanide reduction	
		Absolute rate	Relative rate
	µM	nmoles per min·mg protein	
Succinate	0	292	100
	50	289	99
	100	202	69
Malate	0	402	100
	50	66	16
	100	36	9

ferricyanide reductase is inhibited only 30% at 100 µM. This further confirms the notion that a site of diallate inhibition is at the region between malate and the branching point of the respiratory chain. In order to pin-point the exact site of inhibition, difference spectra of electron transport carriers were taken (Fig. II-12). The top figure, A, is the control, showing absorption peaks of reduced carriers. When 300 uM diallate is added to such mitochondria and aerated, all absorption peaks disappear as illustrated in figure B. Succinate oxidation is not strongly inhibited by diallate (see Figs. II-9 and II-11). If succinate is added to diallate-treated mitochondria, absorption peaks re-appear (figure C). The observation that all carriers are oxidized by diallate treatment strongly suggests that the site of inhibition is towards the substrate site of spectrophotometrically detectable flavoprotein.

The effect of diallate on the activity of malate dehydrogenase was thus examined with a crude enzyme preparation from mitochondria. Enzyme activity was determined by means of NADH oxidation by oxaloacetate. Figure II-13 shows the effect of diallate on malate dehydrogenase activity at two enzyme concentrations. Diallate inhibits the enzyme activity with a half-maximal inhibition at about 120 µM. In view of the small protein concentrations used, this titer is much higher than that expected from the respiration of malate by intact mitochondria. While in this experiment the titer did not differ between two enzyme concentrations, in several other experiments

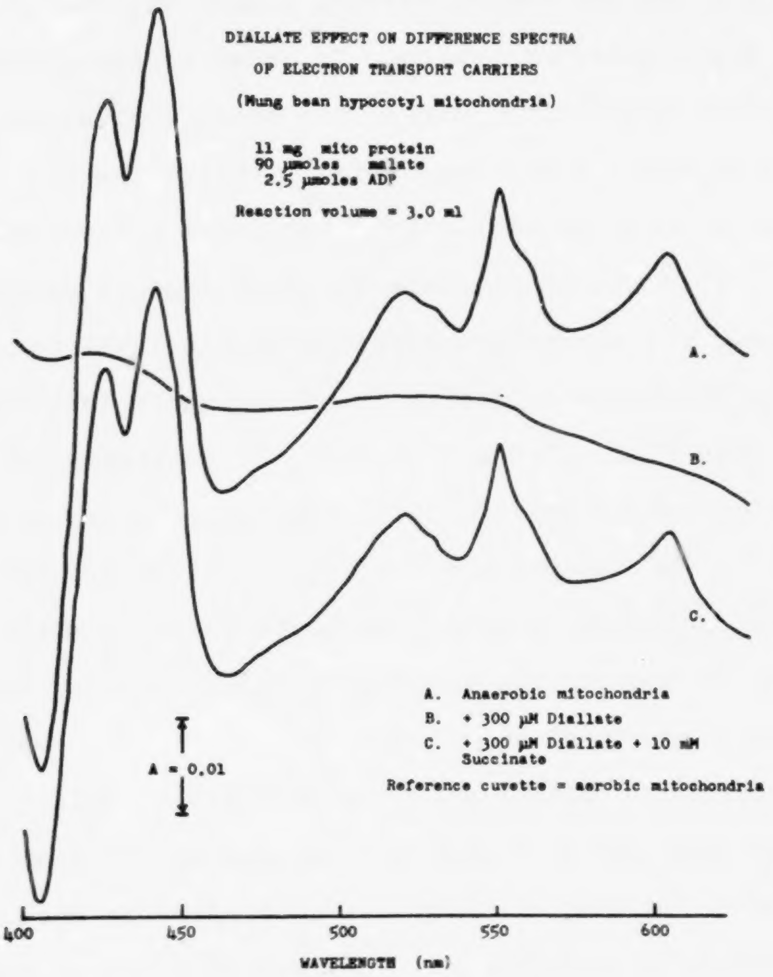
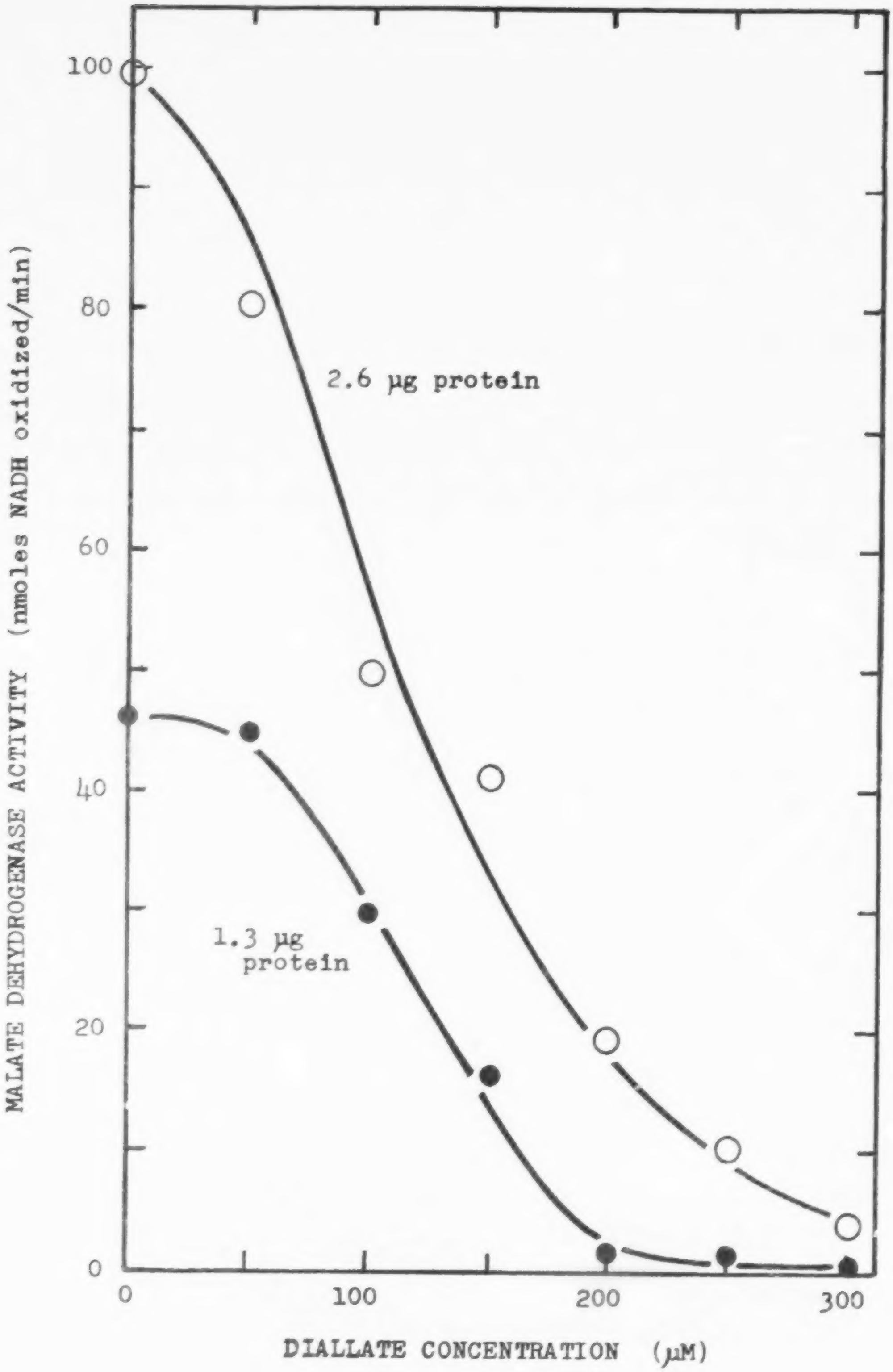


Fig. II-12. Diallate effect on difference spectra of electron transport carriers in mung bean mitochondria.

Fig. II-13. Effect of diallate on malate dehydrogenase activity. Crude preparation of malate dehydrogenase was made from mung bean mitochondria and the activity was examined by means of NADH oxidation with oxaloacetate (see MATERIALS AND METHODS).

DIALLATE EFFECT ON MALATE DEHYDROGENASE



half-inhibition titer values were about 100 µM, but they were not stoichiometrically dependent on the enzyme concentrations used. Diallate inhibition of extracted malate dehydrogenase is reversed by bovine serum albumin (Table II-3), but, as mentioned earlier, diallate inhibition of malate oxidation in intact mitochondria is not reversed by bovine serum albumin. From these studies, diallate inhibition of malate dehydrogenase activity appears to be different from the inhibition of malate respiration by mitochondria.

If titration of diallate is made on state 4 rates, oxidation of different substrates gives different results. The results in Fig. II-14 indicate that malate oxidation is most sensitive (figure A), followed by NADH oxidation (figure C), while succinate oxidation (figure B) is stimulated by diallate below 200 µM and inhibited at 300 µM. The activity of cytochrome oxidase, i.e., ascorbate/TMPD oxidation (figure D), is stimulated by about 30% at concentrations above 100 µM. Different titer values in oxidation of different substrates can be seen on an oxygen electrode trace, as illustrated in Fig. II-15. Malate oxidation is most sensitive, followed by NADH oxidation and succinate oxidation. Cytochrome oxidase activity is not inhibited even at 300 µM.

Inhibition of malate oxidation is not reversed by ADP and m-Cl-CCP. Figure II-16 shows that diallate inhibition of malate oxidation takes place while the mitochondria are in state 4 (figure B), in uncoupled state (figure C), or in state 3 (figure D). Partial reversal of inhibition can be

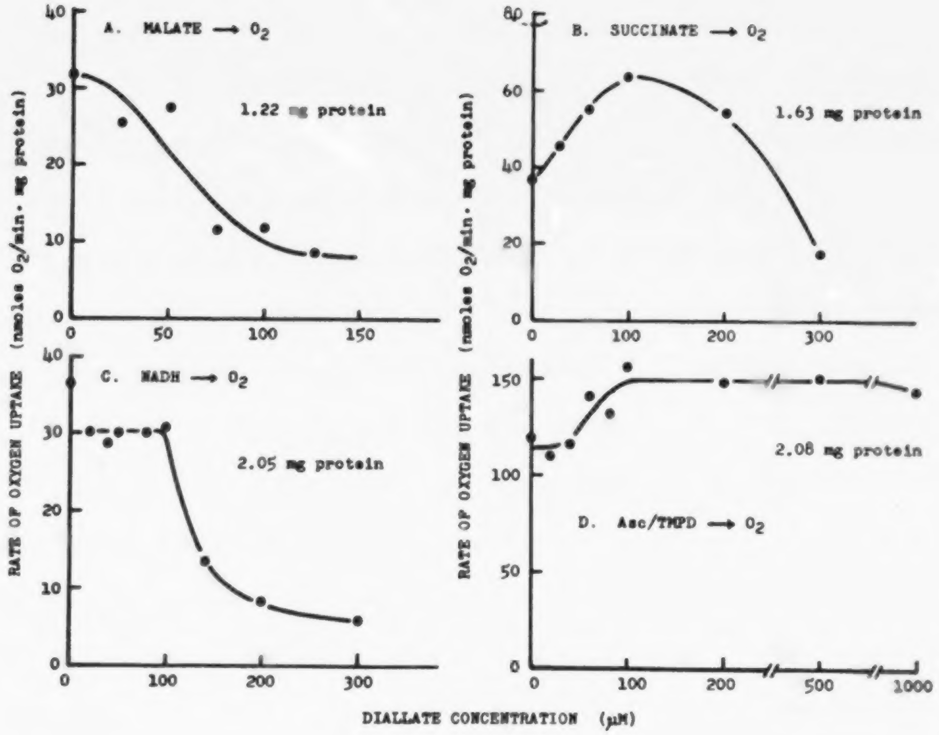


Fig. II-14. Effects of diallate on non-phosphorylating (state 4) rate of malate (A), succinate (B), and NADH (C) oxidation and on cytochrome oxidase activity (D).

For figures A-C, the standard screening procedure (Fig. II-2A) was employed and the rate after diallate addition, but before the addition of the second ADP, was measured. For figure D, the oxidation of ascorbate/TMPD was examined without added ADP, as in Fig. II-2C. Malate = 30 mM; succinate = 10 mM; NADH = 1 mM; ADP = 150 µM.

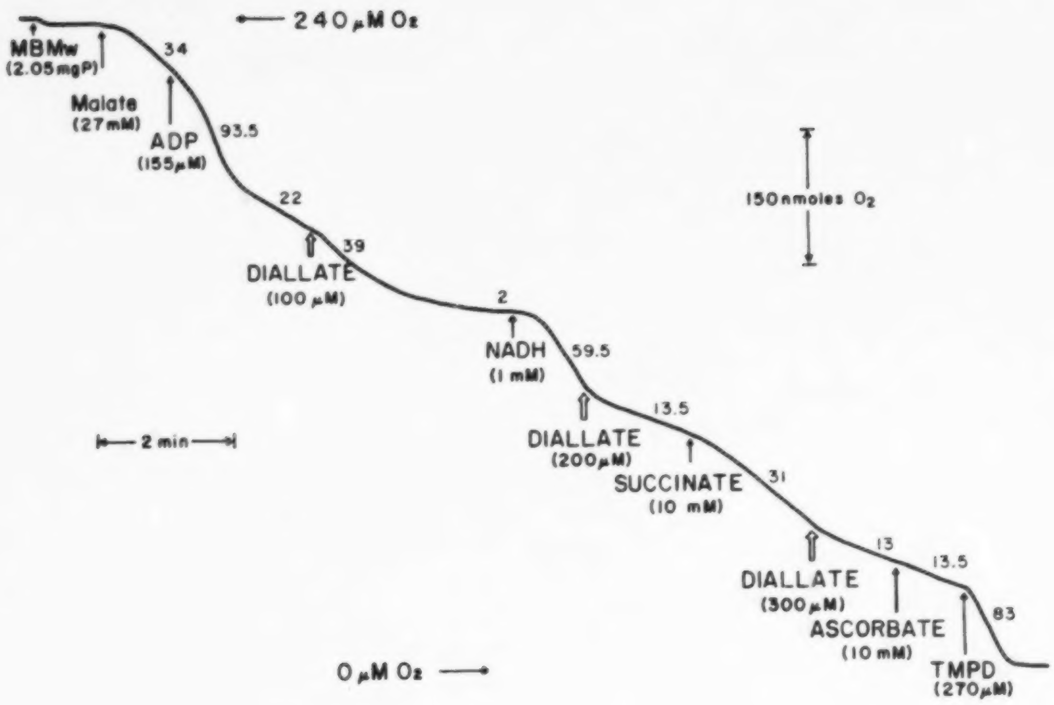


Fig. II-15. An oxygen electrode trace illustrating inhibition of state 4 rate of malate, NADH, succinate, and ascorbate/TMPD oxidation with progressively higher concentration of diallate in mung bean mitochondria. Numbers on the trace are the rates of oxygen uptake in nmoles/min·mg protein. Concentrations in parentheses are the final concentration in the reaction.

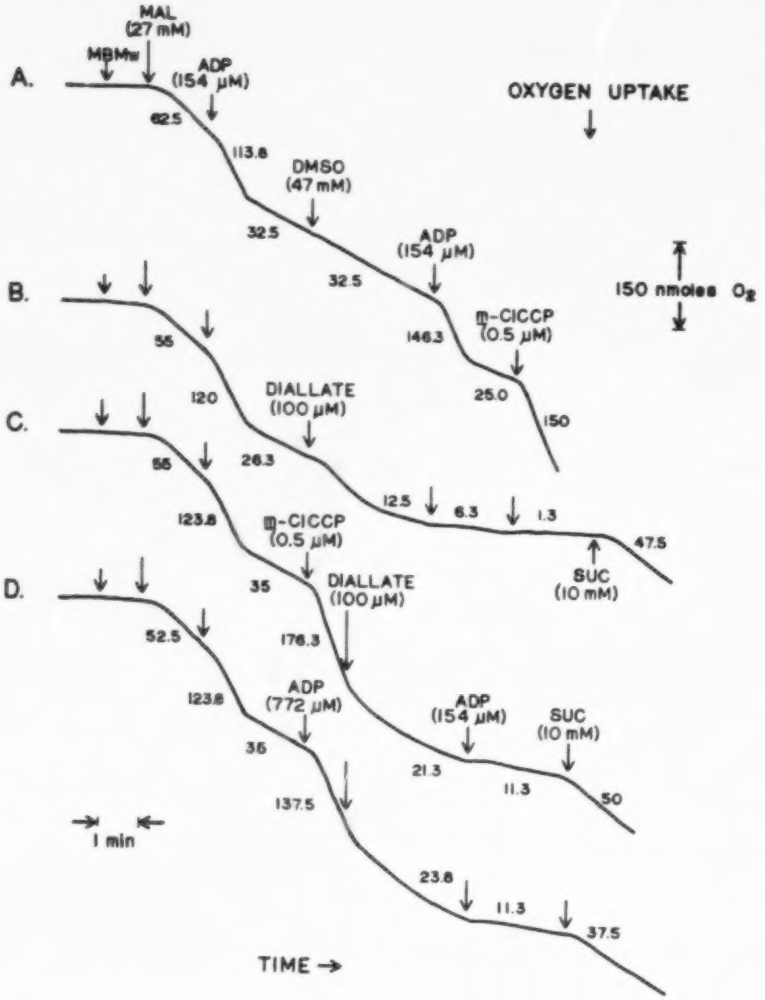


Fig. II-16. Oxygen electrode traces illustrating the effect of diallate on malate oxidation of mung bean mitochondria.

A, control trace, with DMSO but without diallate, showing uncoupling with m-Cl-CCP. B, diallate addition during the second state 4. C, diallate addition during uncoupled state. D, diallate addition during state 3. Arrows without chemicals indicate that the same chemicals as on the traces above were added at the same time of the recording. Numbers on the traces are the rates of oxygen uptake in nmoles/min·mg protein. Concentrations in parentheses indicate the final concentration in the reaction.

achieved by succinate addition (figures B, C, and D; see also Fig. II-11D). It usually takes at least 3 minutes before 100 μM diallate inhibits the state 4 rate maximally. Furthermore, diallate addition during state 4 initially increases the rate of oxygen uptake, particularly when diallate concentrations were higher than 50 μM. These observations prompted us to analyze diallate effect on ATPase. The results in Fig. II-17 shows that ATPase activity, as determined by ADP produced, increases at diallate concentrations of 50 to 100 μM. At higher concentrations, diallate inhibits ATPase activity.

Although diallate effects on the activities of corn root mitochondria have not been presented, the results were practically identical. Perhaps, only slight deviations observed were exact titration values; for example, using the standard screening method with malate as substrate, a titer value of about 200 nmoles diallate/mg protein was obtained. All qualitative aspects were the same as those with mung bean mitochondria.

DISCUSSION

Between mung bean hypocotyl and corn root mitochondria employed in the study, we found no difference in their qualitative responses to herbicides tested. Only small differences were quantitative. The literature indicates that (1) atrazine causes practically no respiratory inhibition

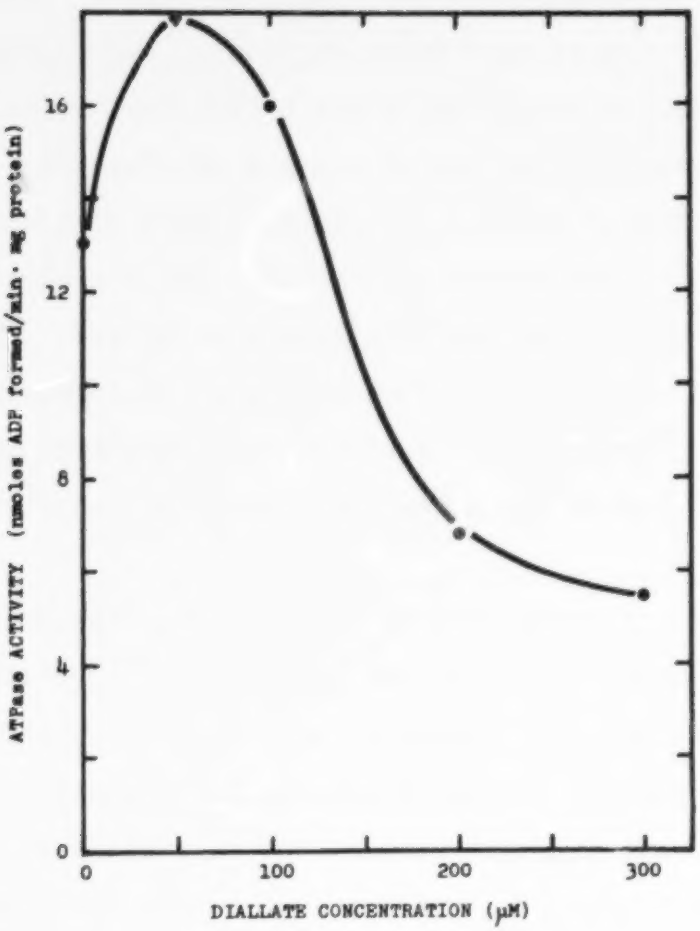


Fig. II-17. Effect of diallate on ATPase activity in mung bean mitochondria.

Mitochondria (0.6 mg protein) were incubated for 5 minutes with 1 mM ATP and ADP formed was measured as in MATERIALS AND METHODS.

of Black Valentine bean hypocotyl mitochondria (Thomson et al., 1974), (2) 2,4-D, 2,4,5-T, propham, and chlorpropham inhibits oxidative phosphorylation of cabbage mitochondria at millimolar concentrations (Lotlikar et al., 1968), (3) trifluralin inhibits succinate state 3 respiration of soybean and mung bean hypocotyl mitochondria (Moreland et al., 1972; Wang et al., 1974), (4) urea herbicides, MBPU and fluometuron, acts to uncouple oxidative phosphorylation of mung bean and soybean mitochondria (Moreland and Boots, 1971; McDaniel and Frans, 1969), and (5) phenolic and nitrile herbicides have been shown to uncouple oxidative phosphorylation of several higher plant and animal mitochondria at about 10^{-5} M (Ashton and Crafts, 1973; Ferrari and Moreland, 1969; Herbicide Handbook, 1974; Moreland and Blackmon, 1970; Moreland et al., 1974; Parker, 1965; Paton and Smith, 1967). These observations in the literature are in good agreement with our findings, and further suggest that higher plant mitochondria, regardless of their source, respond practically identical manner. In addition, preparation of so-called "tightly-coupled" mitochondria from wheat roots, oat and corn shoots, various species of bean hypocotyls, broccoli and cauliflower heads, and white and sweet potatoes have shown basic respiratory properties practically identical to mung bean hypocotyl mitochondria (see Ikuma, 1970, 1972; Bonner, 1965; unpublished observations in the laboratories of W. D. Bonner, Jr. and H. Ikuma). From these considerations, our complete survey with mung bean mitochondria should provide a good basis to evaluate herbicidal phytotoxicity, even though our survey with corn root mitochondria was carried out with only malate as substrate.

Of the seven herbicides which affected mitochondrial activity very little or not at all, atrazine, karbutylate, and simazine are primarily inhibitors of the Hill reaction (see Ashton and Crafts, 1973; Herbicide Handbook, 1974; also next Chapter). Cacodylate and DSMA are pentavalent arsenicals which are generally weak in inhibiting metabolic acitivities (Webb, 1966). Plant mitochondria may not be able to convert them to more potent trivalent forms. Diquat has an oxidation-reduction potential of -349 mV (Ashton and Crafts, 1973) which is close to the potential of NAD, but this herbicide did not cause any effect to malate nor succinate oxidation. Amitrole is considered to be an inhibitor of chlorophyll synthesis (Ashton and Crafts, 1973; Herbicide Handbook, 1974). Lack of effect of these herbicides raises a question as to whether they may not be taken up by the mitochondria. This possiblity was not tested in the present study.

Another group of seven herbicides affected mitochondrial activity at millimolar concentrations. Our results (Table II-1) with two urea-type herbicides (monuron and diuron), two carbamate herbicides (propham and chlorpropham), and two phenoxy herbicides (2,4-D and 2,4,5-T) agree quite favorably with the results reported in the literature on the same or related herbicides (Ferrari and Moreland, 1969; Lotlikar et al., 1968; McDaniel and Frans, 1969; Moreland and Boots, 1971).

Our titer values for uncoupling of oxidative phosphorylation by monuron and diuron is about one to two orders of magnitude higher than those for fluometuron and MBPU. Bromacil is a potent inhibitor of the Hill reaction (Ashton and Crafts, 1973; Hilton et al., 1964; also see next Chapter). As indicated in Table II-1, these herbicides act somewhat differently between succinate and malate oxidation; malate oxidation tends to be more strongly affected. However, relatively higher titer values and their much higher potency in inhibiting the Hill reaction made us not to pursue further on the mode of action these herbicides.

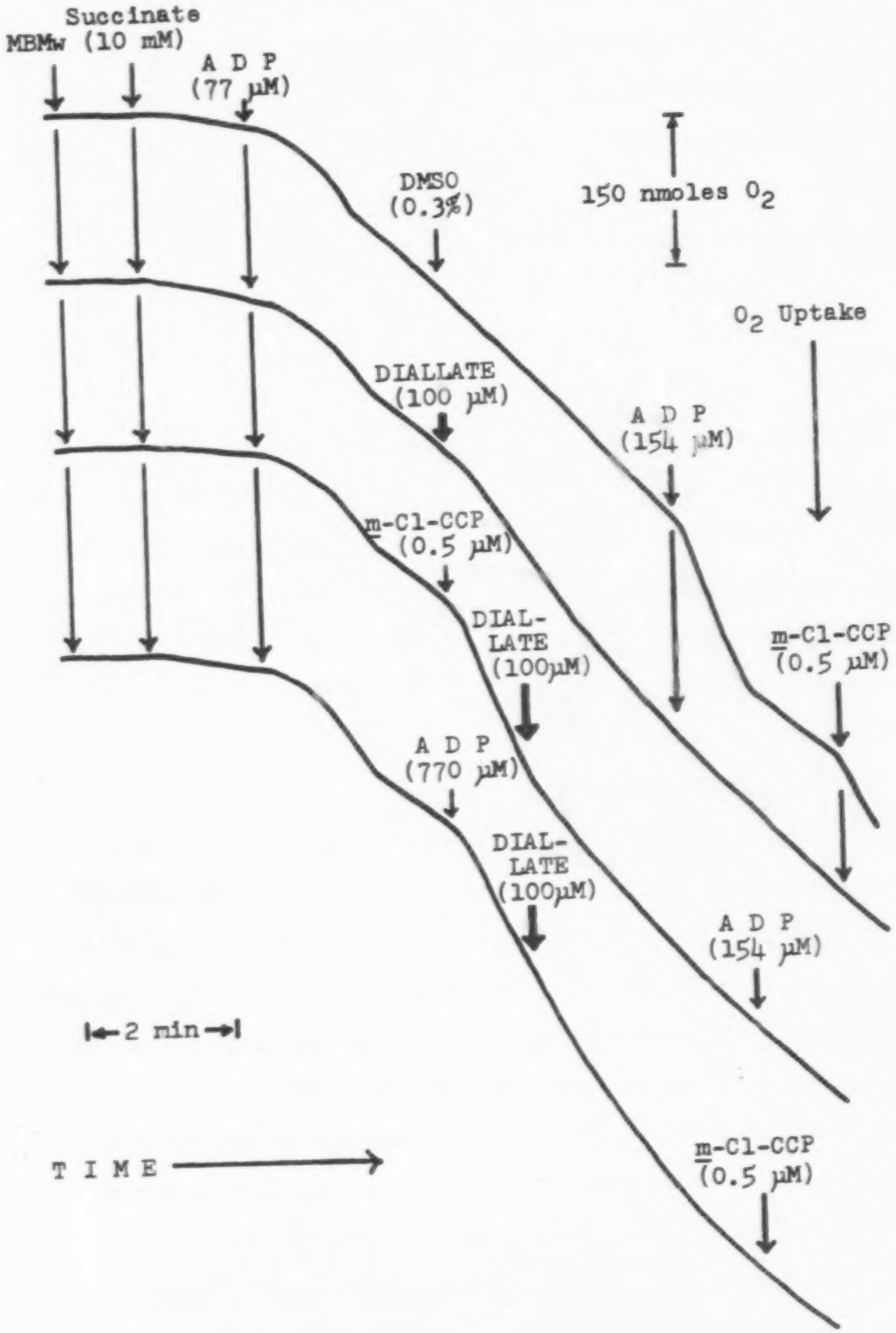
DNOC uncoupled oxidative phosphorylation as expected for phenolic and nitrile herbicides (Ashton and Crafts, 1973; Ferrari and Moreland, 1969; Herbicide Handbook, 1974; Moreland and Blackmon, 1970; Moreland et al., 1974; Parker, 1965; Paton and Smith, 1967). Maximal uncoupling was caused by DNOC at 20-30 μM (Fig. II-3); the concentration is more or less at the same order of magnitude as the titer values of other phenolic herbicides, but about two orders of magnitude higher than m-Cl-CCP (cf. Ikuma and Bonner, 1967b). It is of interest to note that this herbicide does not bind with mitochondria as CCP derivatives (cf. Slater, 1963).

Our titration value of trifluralin for half-maximal inhibition for both succinate and malate oxidation was 20-30 μM (Figs. II-5 and II-6; also see Moreland et al., 1972), while Wang et al. (1974) recently reported a value of 80 μM for succinate oxidation of soybean mitochondria. In addition, they observed that trifluralin inhibition of succinate oxidation was partially reversed by an uncoupler (DNP, nitralin, or oryzalin). We were, however, unable to reverse trifluralin effect with m-Cl-CCP (Fig. II-7). The differences may be due to differences in the source of mitochondria (soybean hypocotyl vs. mung bean hypocotyl or corn root), difference in the osmoticum used for assay (0.15 M mannitol + 0.15 M sucrose vs. 0.5 M mannitol), difference in DMSO concentrations added to the reaction (4% vs. 0.33%), and/or difference in the uncouplers used (DNP, nitralin, or oryzalin vs. m-Cl-CCP). The difference in the uncoupler used may most likely explain the different observations between Wang et al. and us, because m-Cl-CCP binds to mitochondria irreversibly and stoichiometrically while their uncouplers probably do not. It is of interest to note that trifluralin taken up in mitochondria can be washed slowly, presumably due to its high lipid solubility and partition coefficient. If this compound acts as a phosphorylation inhibitor like oligomycin as Wang et al. contend, we would have obtained a difference spectrum showing state 4-5 transition (cf. Chance and Williams, 1956). In repeated attempt, we could not obtain any difference in the spectrum. In addition, titration curves for malate and succinate oxidation were practically identical (Fig. II-5), and there was no detectable effect of this herbicide on cytochrome oxidase activity. All our observations lead to a conclusion that a possible site of action is between the branching point of the respiratory chain and c-type cytochromes. However, DNP-stimulated ATPase activity can be partially inhibited

(Moreland et al., 1972). Obviously more work is needed to resolve conflicting observations.

Diallate is a chemical which shows multiple action. It binds to mitochondria stoichiometrically and irreversibly (Figs. II-9,10, 11). It inhibits only the state 3 respiration of succinate oxidation, while inhibition of malate oxidation is for both states 3 and 4 with the same titer as the succinate state 3 inhibition (Figs. II-10 and II-14). At high concentrations (>100 µM), it inhibits state 4 rates of NADH and succinate oxidation as well as ATPase and malate dehydrogenase activity (Figs. II-13, 14, 17), while cytochrome oxidase activity is slightly stimulated (Fig. II-14). At high concentrations, inhibition of malate oxidation sets is slowly with the initial response of an uncoupling-type (Figs. II-15 and II-16). This initial response may be caused by increased activities of ATPase and cytochrome oxidase (Figs. II-14 and II-17). Subsequent inhibition is not reversed by an uncoupler (Fig. II-15), suggesting that the primary site of inhibition is in the electron transport chain. Studies of difference spectra further suggest that the primary site of inhibition in malate oxidation is towards the substrate side of spectrophotometrically detectable flavoprotein (Fig. II-12). However, in succinate oxidation, the inhibition is primarily on the phosphorylative activity. As shown in Fig. II-18, inhibition of state 3 in succinate oxidation is also not reversed by _m_-Cl-CCP. It thus appears that this herbicide may act on the coupling of phosphorylation with electron transfer in a manner different from oligomycin. Since diallate precipitates out above 100µM,

Fig. II-18. Oxygen electrode traces illustrating the effect of diallate on succinate oxidation of mung bean mitochondria. A. control trace, with DMSO but without diallate, showing uncoupling with m-Cl-CCP. B, diallate addition during the second state 4. C, diallate addition during uncoupled state. D, diallate addition during state 3.


Succinate
MBMw (10 mM)
A D P
(77 µM)
DMSO
(0.3%)
150 nmoles O_2
O_2 Uptake
DIALLATE
(100 µM)
A D P
(154 µM)
m-Cl-CCP
(0.5 µM)
DIAL-
LATE
(100µM)
m-Cl-CCP
(0.5 µM)
A D P
(770 µM)
DIAL-
LATE
(100µM)
A D P
(154 µM)
2 min
T I M E
m-Cl-CCP
(0.5 µM)

we should perhaps consider that the processes which can be inhibited at higher concentrations may be caused secondarily. In view of titration curves, the primary mode of inhibition of diallate must thus be (a) in the inhibition of electron transfer between NAD and flavoprotein, and (b) in the inhibition of phosphorylation.

EVALUATION OF METHOD

Two major sourses of energy supply to any plant cell are oxidative phosphorylation in the mitochondria and photophosphorylation in the chloroplasts. Phytotoxicity of herbicides may likely seen in reduction or inhibition of the capacity of cellular energy supply (see Ashton and Crafts, 1973; Moreland, 1967). In the case of the mitochondria, inhibition of the energy supply can be achieved by (1) inhibiting electron transfer, (2) inhibiting the phosphorylation mechanism, and/or (3) uncoupling of oxidative phosphorylation. Rapid screening of these possibilities can be carried out with our standard screening procedure illustrated in Fig. II-2A. It should, however, be pointed out that the standard procedure slone cannot firmly establish the exact site(s) of phytotoxic effect, as our results amply testify. Thus, depending upon the degree of requirements in search for the biochemical mode of action of a herbicide, our standard method can be viewed sufficient or insufficient.

Our method heavily depends upon the penetration of a herbicide into the isolated mitochondria. If the penetration takes place not at all or very slowly, the method may not detect the phytotoxicity of such a herbicide in mitochondrial functions. In addition, not only from our past year's experience but from others, mitochondria isolated from any plant tissues may respond to a given herbicide practically in an identical manner. We thus strongly feel that our screening method with isolated mitochondria can hardly distinguish selectivity of herbicidal phytotoxicity to given groups of plants. This point can be viewed as an advantage in that a readily available plant tissues can be used as the source of mitochondrial preparation.

From these considerations, the following recommendations can be made:

(1) If the screening of herbicidal phytotoxicity is to assess whether the effect is in the inhibition of electron transfer or phosphorylation, or in uncoupling of oxidative phosphorylation, the procedure illustrated in Fig. II-2A is an ideal procedure.

(2) If a more precise biochemical mode of action should be examined, the above procedure must be supplemented with many other methods, as we attempted in our work.

(3) If selectivity of herbicidal phytotoxicity to various plants must be examined, the recommended procedure with isolated mitochondria cannot provide the answer.

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CHAPTER III

EFFECTS OF HERBICIDES ON CHLOROPLAST PHOTOSYNTHESIS

By Charles F. Yocum and Steven J. Robinson

INTRODUCTION

The process of photosynthesis is comprised of two steps. In the first step, light energy is trapped by chlorophyll and stabilized in the form of chemical energy as adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The second step in the photosynthetic process converts carbon dioxide into sugars enzymatically at the expense of the chemical energy formed in the light. The net reaction consists of release of oxygen by the light reaction and consumption of carbon dioxide by enzymes to produce sugar.

Our current knowledge of the operation of the second step of photosynthesis is well established owing to the work of Calvin and his colleagues (Bassham, 1965), but there are numerous lacunae in our knowledge of the operation of the light reactions. The most recent review (Trebst, 1974) of photosynthetic electron flow and energy conversion presents the current model for these processes. Briefly, the energy conversion apparatus consists of two light reactions or photosystems. One of these, photosystem II, oxidizes water to produce oxygen and a stable, weak reductant. The other photosystem, photosystem I, catalyzes transfer of electrons from a weak oxidant to NADPH, or artificial acceptors such as ferricyanide or methyl viologen. Interposed between the weak reductant of photosystem II and the weak oxidant of photosystem I are a series of electron carriers which include plastoquinone (PQ), cytochrome _f_ (Cyt. _f_) and a copper protein, plastocyanin (PC). Passage of an electron from the reducing side of photosystem II to the oxidizing side of photosystem I through the carriers described above releases an amount of energy sufficient for the synthesis of one molecule of ATP for every pair of electrons traversing this pathway.

Because the photoconversion apparatus of a photosynthetic organism represents in most cases the key to its survival, it is appropriate that assessments of phytotoxicity include studies on photosynthetic electron transport and energy conversion. For the sake of convenience, one can define three types of inhibition which may be observed in appropriate assays of photosynthetic activity (Izawa and Good, 1972). These are:

1. Electron transfer inhibition, caused by an interruption of electron flow at some point in the conversion apparatus;
2. Uncoupling, caused by dissociation of ATP synthesis from electron transfer; and
3. Energy transfer inhibition, caused by an inhibition of ATP synthesis, which in turn slows electron transport. Energy transfer inhibition is reversed by

compounds which act to uncouple electron flow from ATP synthesis.

Izawa and Good (1972) have reviewed the classes of compounds which produce these inhibitions in isolated chloroplasts.

Certain herbicides have played an important role in photosynthesis research. Diuron (DCMU) is a potent inhibitor of photosystem II, and paraquat or methyl viologen is a useful electron acceptor in photosystem I mediated reactions. The inhibitory properties of a number of herbicides, with regard to their effect on photosynthesis, have been investigated (Ashton and Crafts, 1973; Moreland, 1967). Moreland and his colleagues (Moreland et al., 1974; Moreland and Blackmon, 1970; Moreland and Boots, 1971), and Hilton and his coworkers (Hilton et al., 1964) have investigated extensively the effects of a variety of herbicides on photosynthetic energy transduction. In nearly all cases examined, the herbicides tested have been shown to be inhibitors of the Hill reaction (electron donation to ferricyanide from water) and/or photophosphorylation.

In approaching the development of a rapid screening method for phytotoxicity of herbicides, we have attempted to design an experimental system which utilizes a simple procedure (assay of oxygen evolution or oxygen consumption) to assess the effect of a variety of herbicides not only on the Hill reaction, which involves both photosystems, but also their effect on individual photosystems and on the apparatus responsible for ATP synthesis. As will be seen, this approach has allowed us not only to assess phytotoxicity at the level of photosynthetic energy conversion, but has also permitted us in most cases to define with some precision the actual site of inhibition of a herbicide.

MATERIALS AND METHODS

Preparation of Chloroplasts. About 100 g of fresh, deveined spinach or soybean leaves were homogenized for 15 seconds at 4° C in a Waring blendor with 250 ml of a medium containing 0.4 M sucrose, 15 mM NaCl, 20 mM tricine buffer at pH 8.0, and 0.2% bovine serum albumin. The resulting slurry was filtered through 4 layers of cheesecloth and the filtrate was centrifuged at 1000 x *g* for 1 minute. The supernatant was then centrifuged at 5,000 x *g* for 10 minutes and the pellet was resuspended in homogenizing medium and the suspension was centrifuged again for 10 minutes at 5,000 x *g*. The pellet from this step were resuspended in a small volume of homogenizing medium at a chlorophyll concentration of about 2 mg/ml. About 30 mg of chlorophyll are usually obtained by this method from 100 g spinach leaves. The resuspended chloroplasts were stored at -70° in 0.5 ml aliquots until use. Chlorophyll content was determined by the procedure of Arnon (1949).

Standard Screening Methods with an Oxygen Electrode. Three major screening methods used in this study are:

(1) Examination of non-cyclic electron transport from water through the two photosystems with ferricyanide as the electron acceptor;

(2) Analysis of photosystem II with phenylenediamine (PD) or dimethylquinone (DMQ) plus ferricyanide as the electron acceptor; and

(3) Analysis of photosystem I with ascorbate as the electron donor, dichlorophenol indophenol (DPIP) as the electron mediator, and methylviologen (MeV) and the electron acceptor.

Similar to the mitochondrial study, herbicides are expected (a) to inhibit electron transfer, (b) to inhibit phosphorylation, and/or (c) to uncouple phosphorylation. Figure III-1 presents in schematic form the expected results, in terms of oxygen electrode traces, obtainable with the screening methods (1) and (3). This figure is analogous to Fig. II-2 for the mitochondrial study, and the encircled numbers denote four possible types of herbicidal effects; i.e., the line ① refers to no effect by herbicide, line ② indicates that the herbicide acts to uncouple phosphorylation from electron transfer, line ③ is the trace for inhibition of electron transfer by the herbicide, and line ④ shows herbicidal inhibition of phosphorylation. The second screening method is identical to the method 1, except that methylamine is replaced by PD or DMQ. In this assay, PD or DMQ normally stimulates the rate of oxygen evolution as in line ① of Fig. III-1A, while herbicidal inhibition of photosystem II

Fig. III-1. Expected recorder traces showing possible modes of action of herbicides.

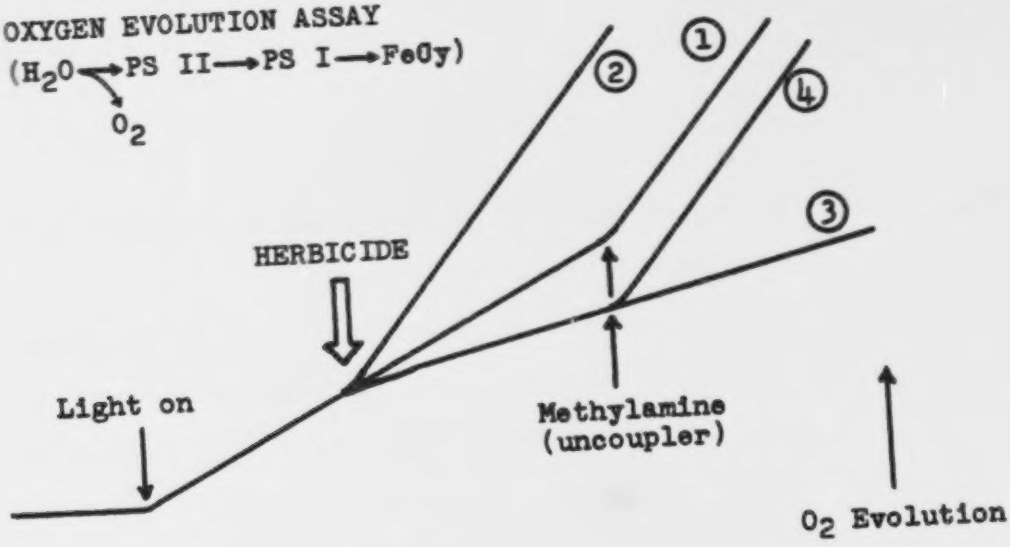
Encircled numbers on each trace indicate, as follows: ①, no effect of herbicide or the same as control; ②, herbicide acting as an uncoupler; ③, herbicide acting as an inhibitor of electron transfer; ④, herbicide acting as an inhibitor of phosphorylation.

A, the oxygen evolution assay for non-cyclic electron flow through photosystems II and I to ferricyanide (FeCy).

B, the oxygen consumption assay detecting photosystem I activity with ascorbate as the electron donor, dichlorophenolindophenol (DPIP) as an electron mediator, methyl viologen (MeV) as another electron mediator between photosystem I and oxygen, and oxygen as the final electron acceptor. In the latter assay, 7 µM DCMU was present in the assay medium to block the photosystem II activity.

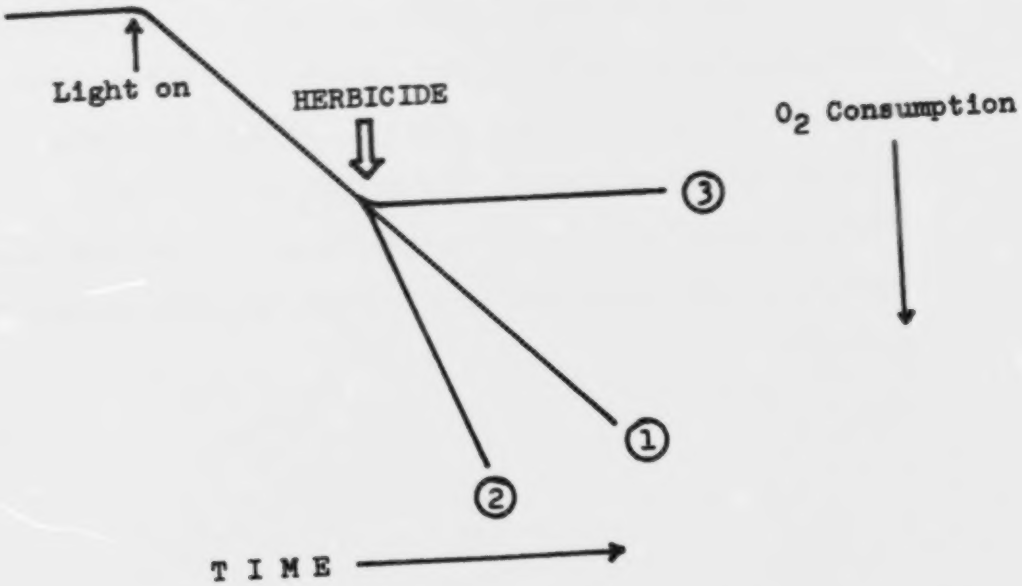
A. OXYGEN EVOLUTION ASSAY

$(H_2O \rightarrow PS\ II \rightarrow PS\ I \rightarrow FeCy)$, $\searrow O_2$


②
①
④
③
HERBICIDE
Light on
Methylamine (uncoupler)
O_2 Evolution

B. OXYGEN CONSUMPTION ASSAY

$(Asc./DPIP \rightarrow PS\ I \rightarrow MeV \rightarrow O_2)$


Light on
HERBICIDE
O_2 Consumption
③
①
②
T I M E

electron transport is expected to show the result shown in line 3 .

Oxygen evolution or consumption is measured in a thermostatted glass cuvette of 1.6 ml capacity (Gilson Biomedical Research, Madison, Wisconsin) with an oxygen electrode (Yellow Springs Instrument, Yellow Springs, Ohio) which was connected to a home-made circuit box and a Heath Schlumberger recorder Model SR-255-B. The light source was an Oriel tungsten-iodine lamp which provided a saturating dose of white light of 10^6 to 10^8 ergs per cm^2 per second. The medium for oxygen evolution study was composed of 12 mM tricine buffer at pH 8.0, 60 mM NaCl, 2.4 mM potassium ferricyanide, and chloroplast suspension with 10-20 μg chlorophyll. When photosystem II electron transport was assayed, 0.25 mM PD or DMQ was added to the reaction mixture. For the assay of photosystem I, 60 mM ascorbate, 6 μM DPIP, 10 μM MeV, and 7 μM DCMU replaced the ferricyanide in the above reaction mixture. In this system, oxygen consumption, rather than evolution, was measured as in Fig. II-1B.

Because of relative insolubility of many herbicides, herbicide stock solutions were prepared in DMSO or ethanol or water. A microliter quantity was added to the reaction cuvette each time. Basal electron flow was measured for 1 minute after herbicidal addition. Uncoupling of photophosphorylation was carried out by an addition of methylamine at 20 mM.

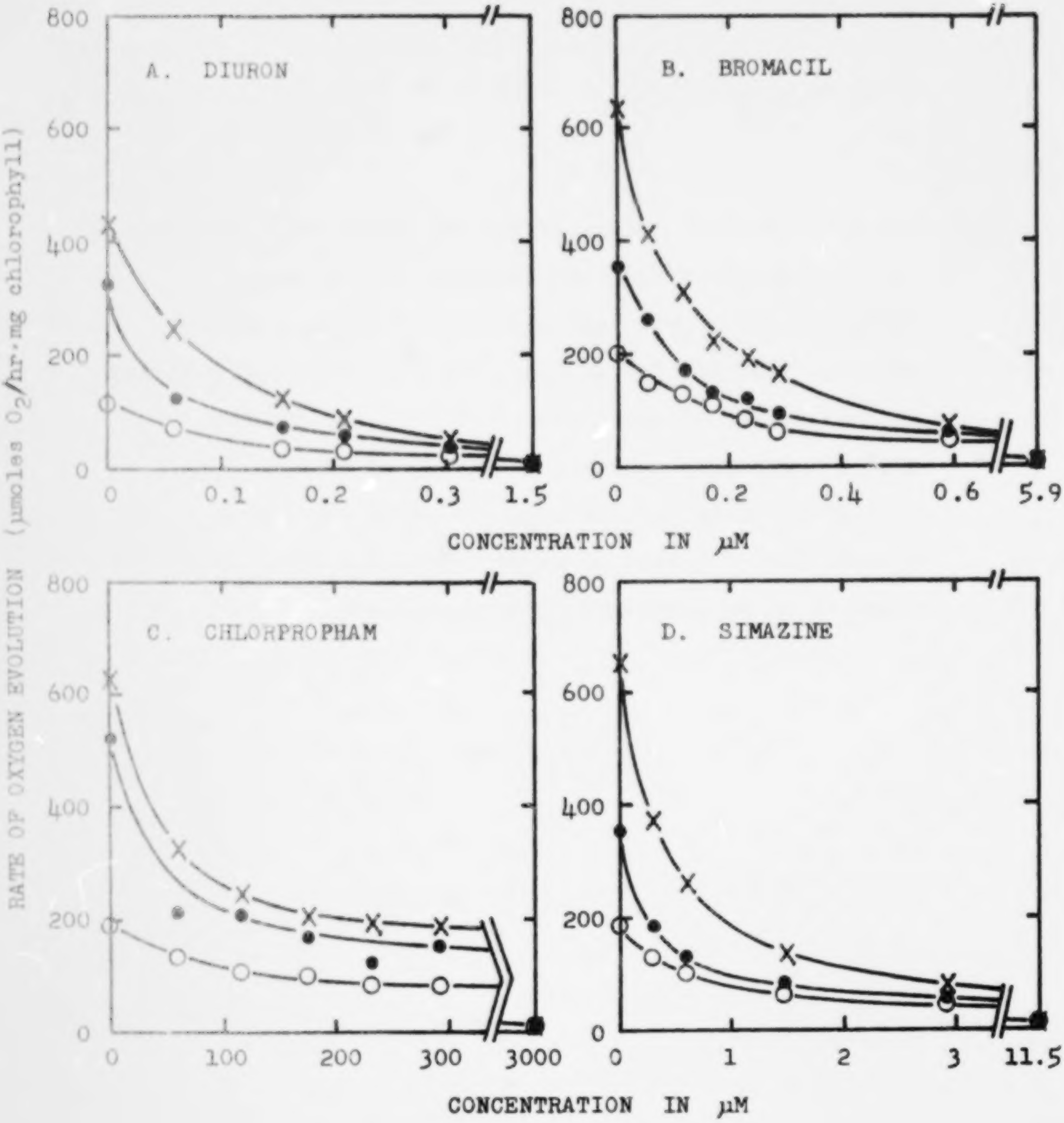
Analysis of Photophosphorylation. When a herbicide was suspected to act as an inhibitor of phosphorylation from the oxygen electrode assays, photophosphorylation activity was assayed according to the Avron procedure (Avron, 1960). In addition to the basic constituents, the reaction mixture contained 10^6 CPM orthophosphate-32. Illumination times were 1 minutes for both cyclic and non-cyclic assays. Activity was determined by gas-flow counting (with a Model 181 counter of Nuclear Chicago) of aliquots of the reaction mixture after extraction with isobutanol-benzene-water.

RESULTS

General Survey of Effects of Seventeen Herbicides. Using the oxygen evolution methods (1 and 2), herbicidal effects were examined for all 17 herbicides up to millimolar concentrations. Three herbicides (cacodylate, diquat, and DSMA) did not affect the activity of chloroplasts at all. Most of the remaining herbicides inhibited photosynthetic electron transport at photosystem II. The effects of 4 herbicides are illustrated in Fig. III-2. Chlorpropham, bromacil, and simazine inhibit chloroplast activity in a manner identical to diuron or DCMU; they inhibit ferricyanide reduction in the presence or absence of PD and methylamine. Other herbicides tested which showed similar behavior were atrazine, 2,4-D, DNOC, Karbutylate, monuron, propham, and 2,4,5-T.

Another indication that the above mentioned herbicides inhibit photosystem II activity is in the fact that all these herbicides did not inhibit photosystem I electron transfer

Fig. III-2. Titration of the effect of diuron (A), bromacil (B), chlorpropham (C), and simazine (D) on oxygen evolution (Hill reaction) activity of spinach chloroplasts. Open circles indicate the Hill reaction activity in the absence of uncoupler or PD. Closed circles denote the oxygen evolution activity after additions of 0.25 mM PD in the ferricyanide reduction assay. X marks show the rate of oxygen evolution after additions of 20 mM methylamine (uncoupled rate).



A. DIURON
B. BROMACIL
C. CHLORPROPHAM
D. SIMAZINE
RATE OF OXYGEN EVOLUTION (μmoles O_2/hr·mg chlorophyll)
CONCENTRATION IN μM
CONCENTRATION IN μM
0
200
400
600
800
0
0.1
0.2
0.3
1.5
0
0.2
0.4
0.6
5.9
0
100
200
300
3000
0
1
2
3
11.5

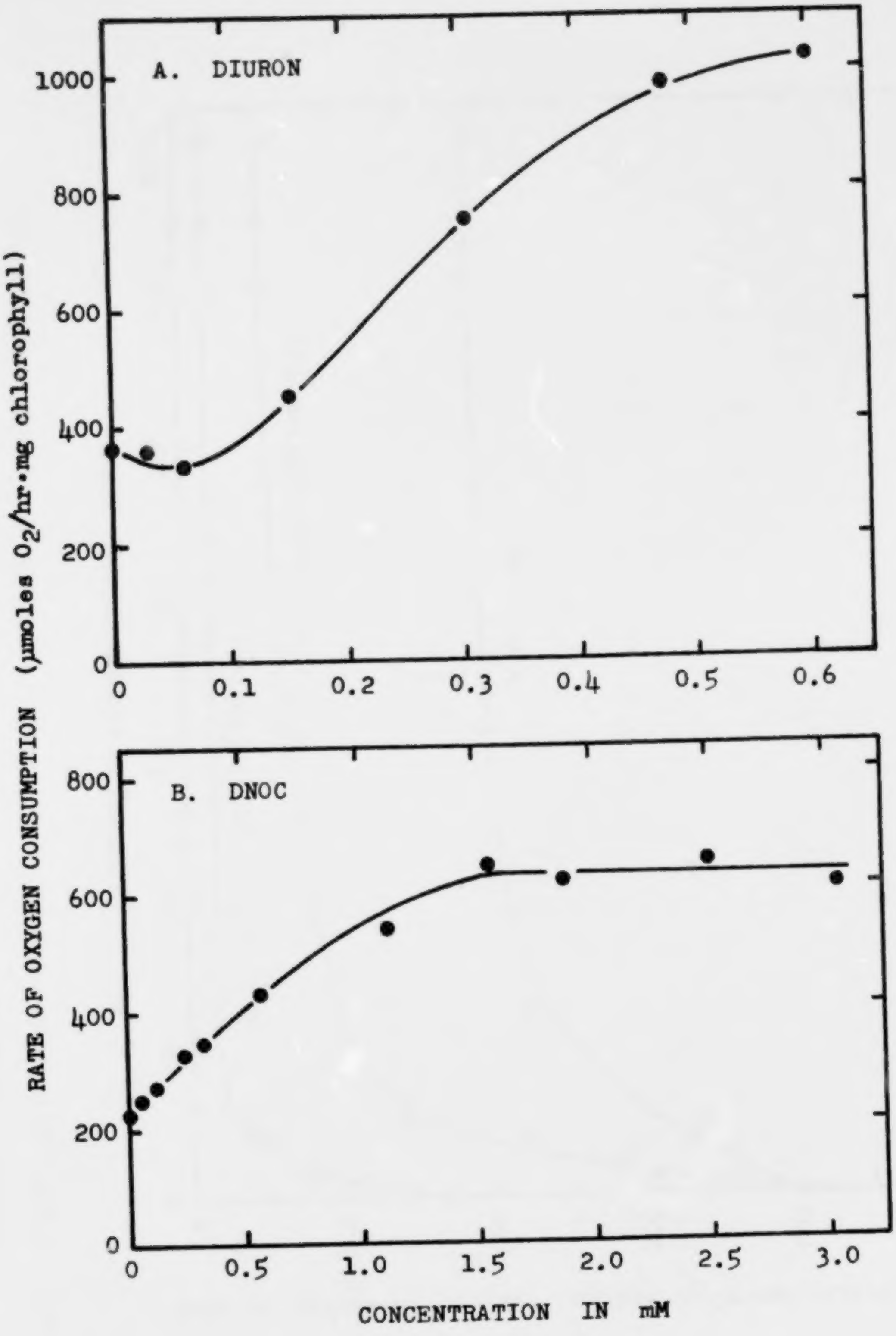
activity in the oxygen consumption assay. With diuron and DNOC, the rate of oxygen consumption is in fact stimulated about three fold at concentrations about 1,000 times those required to inhibit photosystem II activity (Fig. III-3).

Figure III-4 presents the effect of trifluralin on chloroplast electron flow. This herbicide is also an inhibitor of electron flow when ferricyanide serves as the terminal electron acceptor. Note, however, that addition of PD to the assay medium attenuates toxicity. This result indicates that the site of trifluralin inhibition differs from that of diuron; trifluralin must inhibit electron transfer at a point between the two light reactions, not at the diuron-sensitive site. This conclusion is supported by the observation that this herbicide has no effect on photosystem I activity.

Figure III-5 shows the effect of diallate on electron transfer activity of spinach chloroplasts. These titration curves indicate that diallate is an inhibitor of both non-cyclic electron flow through two photosystems and photosystem II activity catalyzed by PD. Careful examination of this figure shows, however, that different concentrations of diallate are required to produce a half-maximal inhibition in the three assay systems used. These concentrations or I_{50} values are 15 µM for coupled chloroplast activity, 40 µM for uncoupled chloroplast activity, and 70 µM for the assay with PD. The I_{50} shift with uncoupling suggests that diallate is a unique herbicide in that it may act as an energy transfer

Fig. III-3. Titration of diuron (A) and DNOC (B) effects on photosystem I activity of isolated spinach chloroplasts.

The oxygen consumption assay illustrated in Fig. III-1B was employed in these studies. Methylamine uncoupled the photosystem I activity; methylamine uncoupled rates were 1310 ± 150 umoles O_2/hr·mg chlorophyll for the diuron experiments and 840 ± 32 umoles O_2/hr·mg chlorophyll for DNOC experiments.


A. DIURON
B. DNOC
RATE OF OXYGEN CONSUMPTION (µmoles O2/hr·mg chlorophyll)
1000
800
600
400
200
0
0
0.1
0.2
0.3
0.4
0.5
0.6
800
600
400
200
0
0
0.5
1.0
1.5
2.0
2.5
3.0
CONCENTRATION IN mM

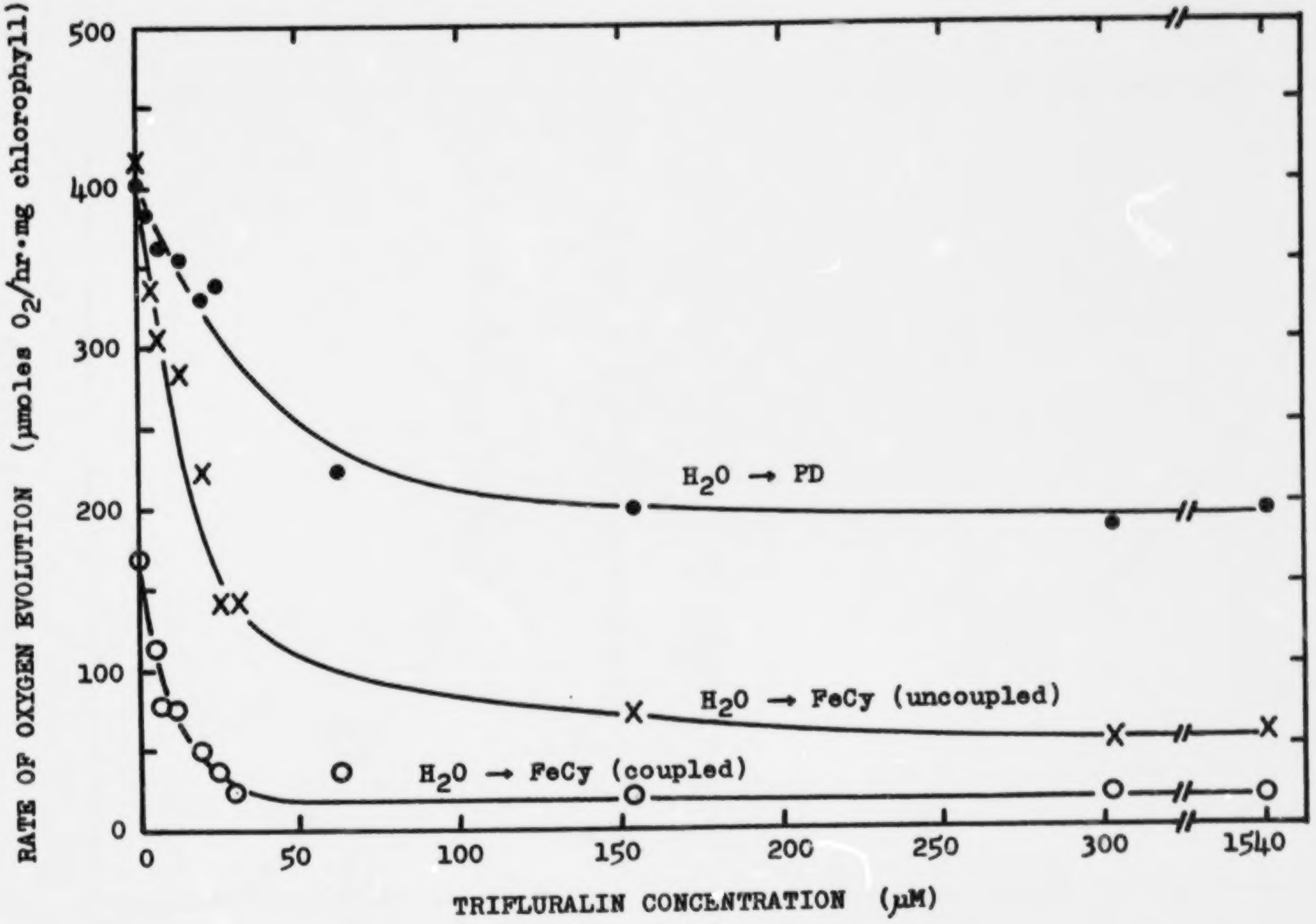


Fig. III-4. Titration of trifluralin effect on oxygen evolution activity of spinach chloroplasts.

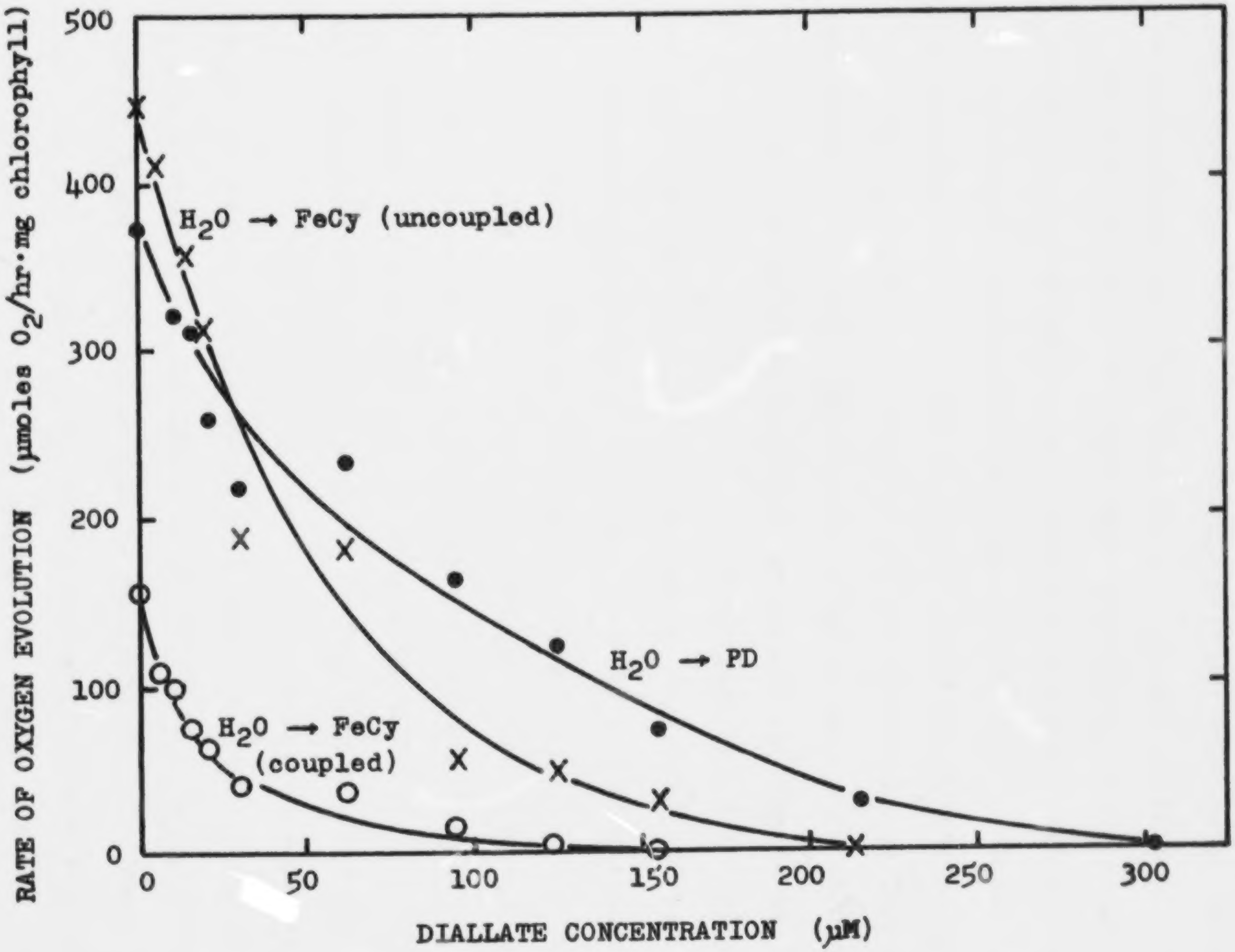


Fig. III-5. Titration of diallate effect on oxygen evolution activity of spinach chloroplasts.

inhibitor, while the PD assay shows it to be a weak photosystem II inhibitor.

Titration experiments similar to those shown in Figs. III-2 to III-5 were carried out with all seventeen herbicides. Table III-1 lists the I_{50} values obtained. An examination of this table shows nearly all herbicides to be inhibitors of photosynthetic electron transport, although the concentrations required for half-maximal inhibition vary depending on the herbicide employed. Diuron, karbutylate, simazine, atrazine, and bromacil are all capable of producing 50% inhibition at concentrations below 1 μM. The other herbicides are less effective, requiring concentrations up to 3 mM (amitrole) for 50% inhibition.

Similar titration curves were examined using soybean chloroplasts to see if an alteration in inhibition occurred with chloroplasts from another plant species. The results of these experiments, given in Table III-2, indicate that the results are practically identical. Amitrole, a weak inhibitor in spinach chloroplasts, did not show any effect in soybean chloroplasts, and titer values for other herbicides also changed slightly.

It should be mentioned that no herbicide tested inhibited the photosystem I activity up to a millimolar concentration range examined. In addition to diuron and DNOC, monuron acted to uncouple photosystem I activity; chlorpropham, diquat, and propham exerted slight uncoupling at millimolar concentrations; and the rest of herbicides did not act as uncouplers.

Table III-1. Half-maximal inhibition values (I_{50} values) of herbicides in the oxygen evolution assay ($H_2O \longrightarrow$ PS II $\longrightarrow$ PS I $\longrightarrow$ FeCy) with isolated spinach chloroplasts.

HERBICIDE	I_{50} Value	Remarks
Amitrole	3 mM	
Atrazine	0.2—0.3 µM	
Bromacil	0.2—0.3 µM	
Cacodylate	- - -	No effect at 3 mM
Chlorpropham	150—200 µM	
2,4-D	1.5 µM	
Diallate	20 µM	
Diquat	- - -	No effect at 3 mM
Diuron	0.1 µM	
DNOC	15 µM	
DSMA	- - -	No effect at 2.5 mM
Karbutylate	0.1 µM	
Monuron	1 µM	
Propham	150—200 µM	
Simazine	0.5—0.6 µM	
Trifluralin	10—20 µM	
2,4,5-T	15—20 µM	

Table III-2. I_{50} values of herbicide in the oxygen evolution assay with isolated soybean chloroplasts.

HERBICIDE	I_{50} Value	Remarks
Amitrole	- - -	No effect at 3 mM
Atrazine	0.5—1.5 µM	
Bromacil	0.2—0.3 µM	
Cacodylate	- - -	No effect at 3 mM
Chlorpropham	150—250 µM	
Diallate	50 µM	
Diquat	- - -	No effect at 3 mM
Diuron	0.1—0.2 µM	
DNOC	30—60 µM	
DSMA	- - -	No effect at 3 mM
Karbutylate	0.1 µM	
Monuron	1 µM	
Propham	300—500 µM	
Simazine	1—2 µM	
Trifluralin	25—50 µM	
2,4,5-T	0.5—1 mM	

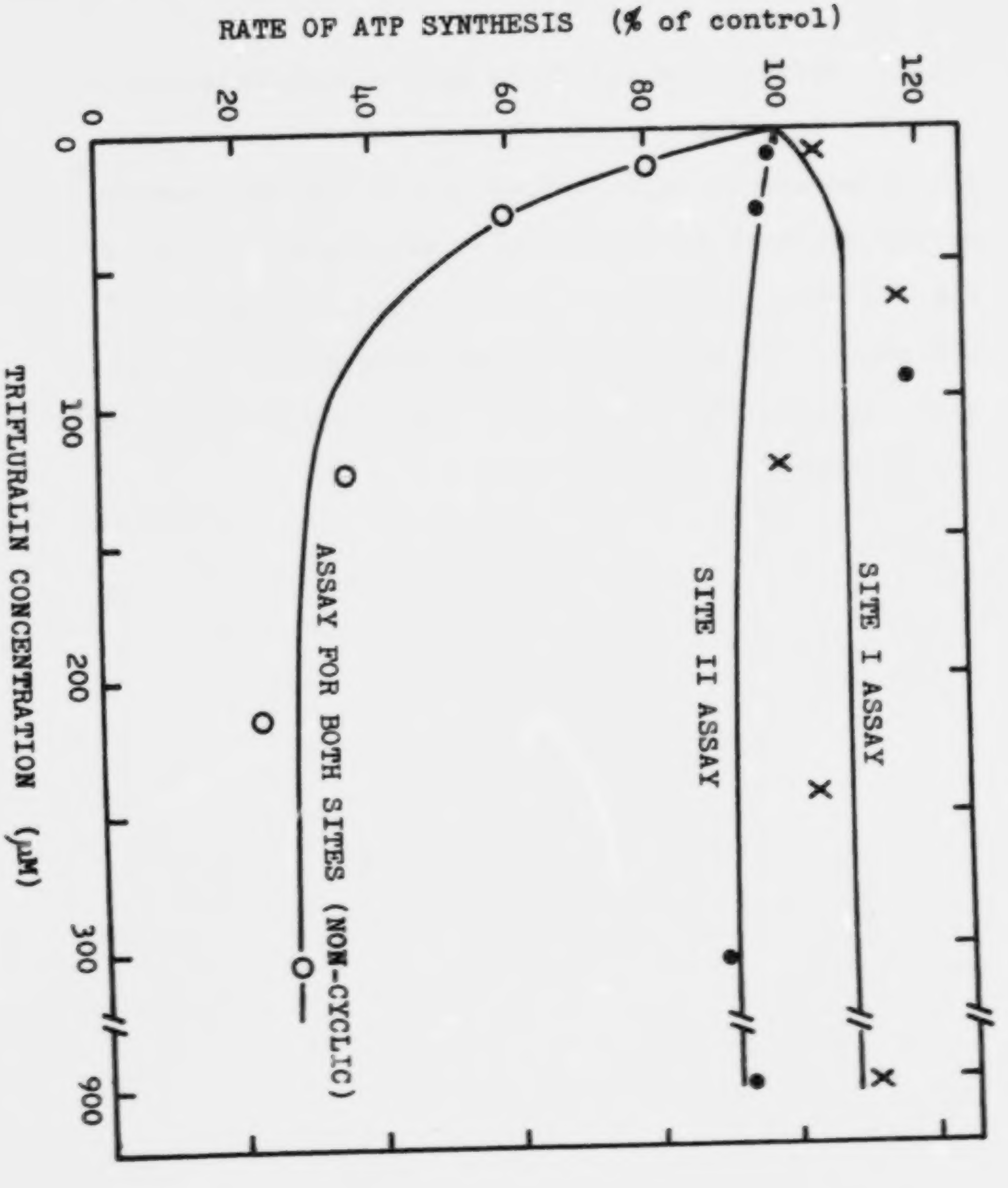
Effects of Trifluralin and Diallate on Photophosphorylation in Spinach Chloroplasts. Trifluralin and diallate inhibited photosynthetic electron transfer in a manner different from the rest of herbicides tested; i.e., at a site different from the diuron inhibition site. Furthermore, the results with diallate suggested that this herbicide may act as an inhibitor of phosphorylation. We have, therefore, examined the effects of these herbicides on phosphorylative activity of isolated spinach chloroplasts.

Figure III-6 shows the titration for trifluralin inhibition of photophosphorylation. This herbicide blocks non-cyclic phosphorylation quite effectively, but neither site I nor site II phosphorylation is inhibited. In these experiments, the phosphorylation efficiency or P/e_2 ratio held fairly constantly throughout. These results lead us to a conclusion that the site of trifluralin inhibition is not closely associated with either site I or site II of photophosphorylation, but the inhibition takes place somewhere between the two photosystems.

Figure III-7 illustrates the titration of diallate effect to inhibit photophosphorylation. Non-cyclic phosphorylation is effectively inhibited at low concentrations of diallate, while site II phosphorylation is inhibited only at significantly higher concentrations and site I phosphorylation is not inhibited at all. Apparent stimulation of phosphorylation at site I in this particular experiment is the result of stimulation of electron transport. The phosphorylation efficiency

Fig. III-6. Effect of trifluralin on photophosphorylation activity of spinach chloroplasts.

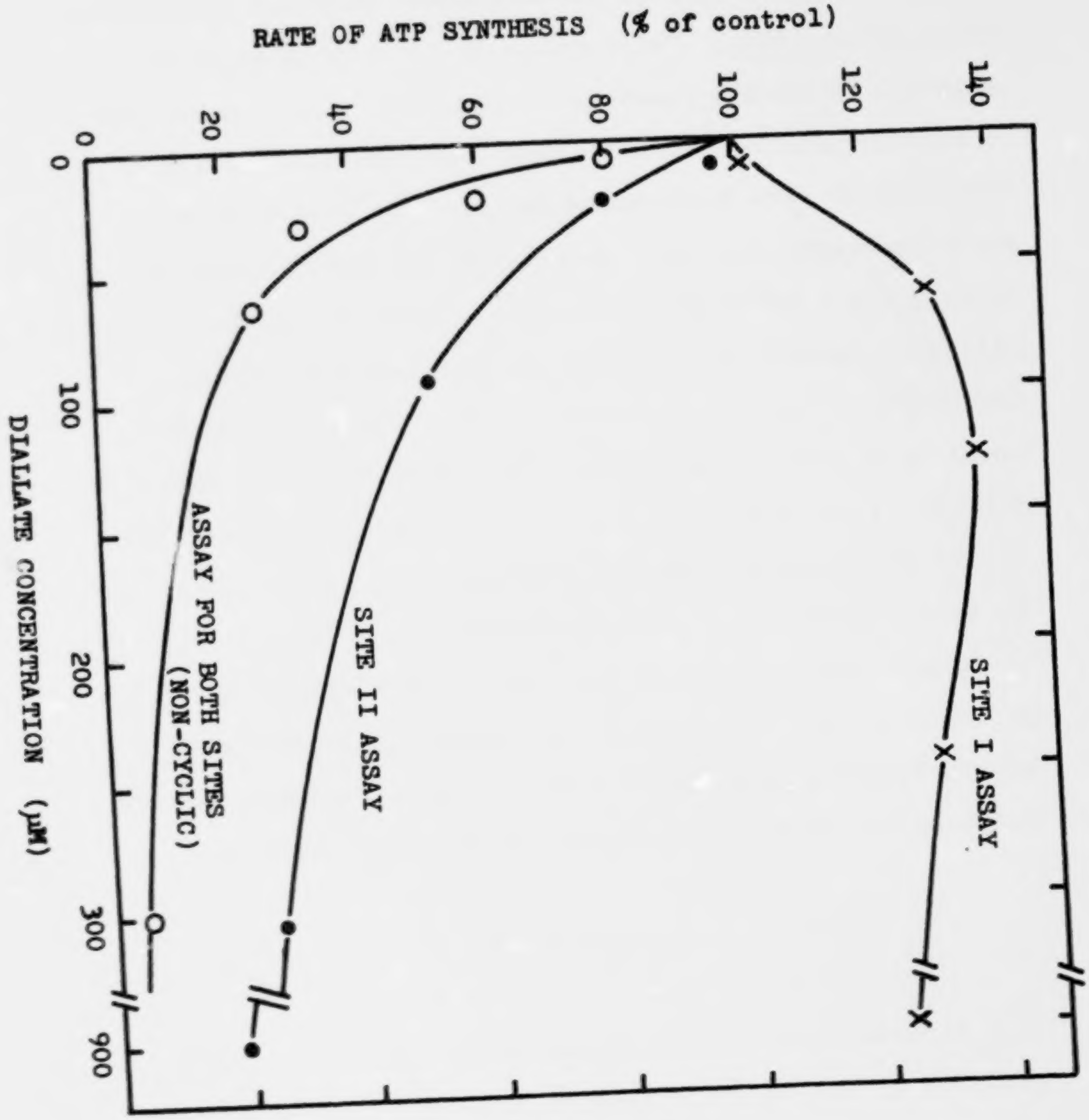
The site I assay was similar to the oxygen consumption assay of Fig. III-1B; i.e., Asc./DAD→PS I→MeV→O_2, which gave the control rate of 440 μmoles ^{32}P esterified per hour per mg chlorophyll. The site II assay was H_2O→PS II→DMQ, the control rate of which was 208 μmoles ^{32}P esterified per hour per mg chlorophyll. The assay for both sites were carried out in the same manner as shown in Fig. III-1A; the control rate was 270 μmoles ^{32}P esterified per hour per mg chlorophyll.



RATE OF ATP SYNTHESIS (% of control)
0
20
40
60
80
100
120
TRIFLURALIN CONCENTRATION (μM)
0
100
200
300
900
SITE I ASSAY
SITE II ASSAY
ASSAY FOR BOTH SITES (NON-CYCLIC)

Fig. III-7. Effect of diallate on photophosphorylation activity of spinach chloroplasts.

The three assays were carried out in the same manner as for trifluralin experiments (see figure legend of Fig. III-6). The control rates were as follows: 400 µmoles ^{32}P esterified/hr·mg chlorophyll for the site I assay, 470 for site II assay, and 268 for sites I + II assay.


RATE OF ATP SYNTHESIS (% of control)
0
20
40
60
80
100
120
140
0
100
200
300
900
DIALLATE CONCENTRATION (μM)
SITE I ASSAY
SITE II ASSAY
ASSAY FOR BOTH SITES
(NON-CYCLIC)

or P/e_2 was constant throughout. Thus, these results are in good agreement with the results from oxygen electrode studies (cf. Fig. III-5).

Figure III-8 depects the effects of trifluralin and diallate on cyclic photophosphorylation mediated by phenazine methosulfate (PMS). Trifluralin is not effective as an inhibitor of cyclic phosphorylation. Since this herbicide apparently does not affect either phytosystem I electron transport or site I phosphorylation, this observation is consistent with the idea that cyclic phosphorylation is largely a system of electron flow around photosystem I. Diallate presents a different picture, causing strong inhibition of cyclic phosphorylation. Diallate does not chemically interact with PMS. This herbicide does not also inhibit either phosphorylation associated with site I (Fig. III-7) or electron transport through photosystem I. It may be that PMS-mediated cyclic phosphorylation cannot rigorously be equated with a pure photosystem I reaction, or that PMS-mediated cyclic phosphorylation includes the portion of phosphorylation associated with electron transport between photosystems which is sensitive to diallate inhibition.

DISCUSSION

Soybean chloroplasts showed lower (about one half) activity of the oxygen evolution activity ($H_2O \longrightarrow FeCy$) and lower (about one half) activity of coupling (examined with 20 mM methylamine addition) than spinach chloroplasts. In

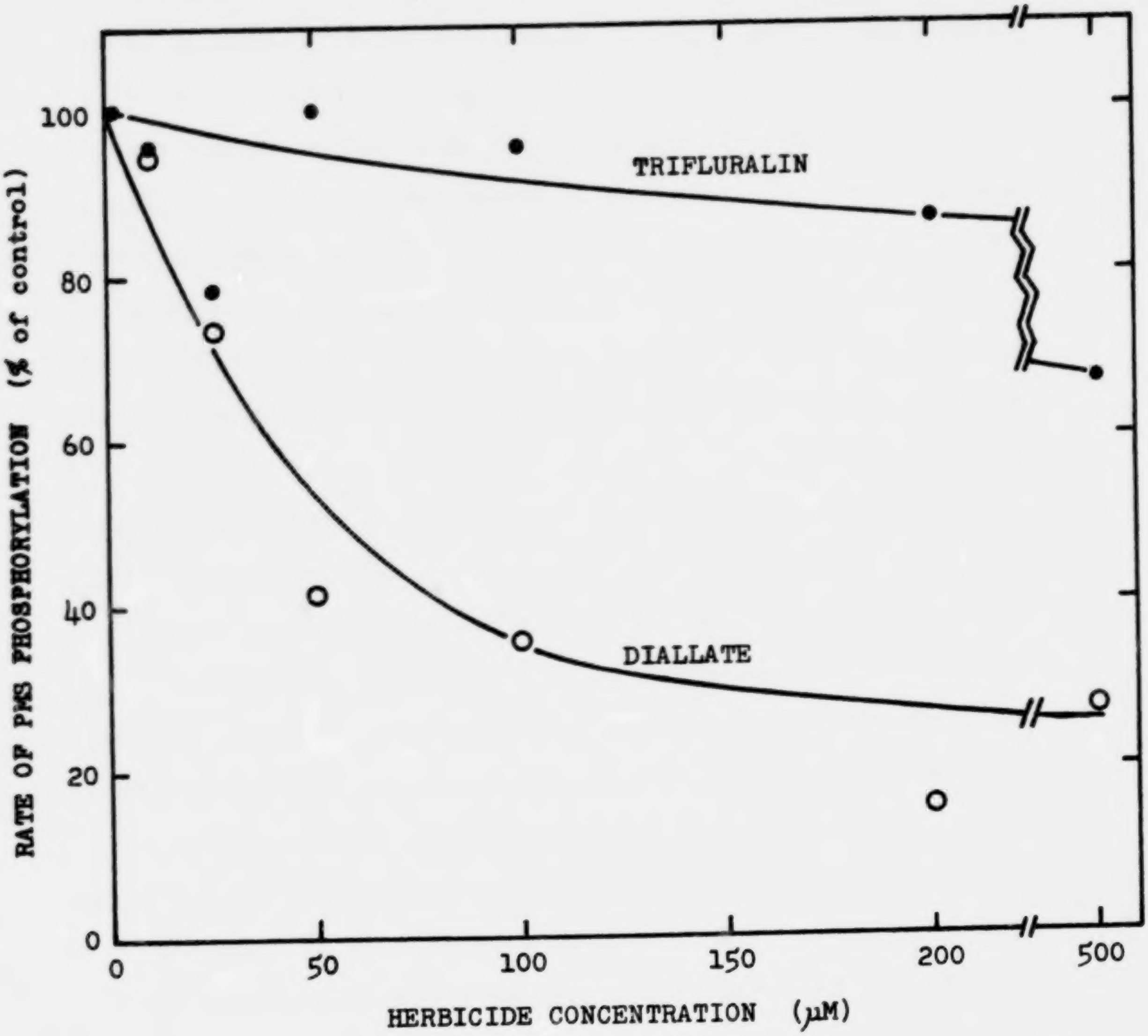


Fig. III-8. Effects of trifluralin and diallate on cyclic photophosphorylation in spinach chloroplasts.

Cyclic photophosphorylation mediated by phenazine methosulfate (PMS) was examined. Control phosphorylation rates were 801 µmoles ^{32}P esterified/hr·mg chlorophyll for trifluralin treatment and 1,322 for diallate treatment.

the oxygen consumption assay of photosystem I activity ($asc/DPIP \longrightarrow PS\ I \longrightarrow MeV \longrightarrow O_2$), the activity of soybean chloroplasts was about one-third less than that of spinach chloroplasts. Nevertheless, the results obtained with soybean chloroplasts (Table III-2) were practically identical with those obtained with spinach chloroplasts (Table III-1). Slight differences observed were quantitative rather than qualitative. In the study of phytotoxicity of herbicides on chloroplast activity, the source of chloroplasts may not play an important role. Spinach chloroplasts whose physiological and biochemical characterization has been extensively studied should serve as a good experimental material for this type of study.

Of the seventeen herbicides tested, cacodylate, diquat, and DSMA showed no effect and amitrole very weak effect on electron transport activity. Amitrole apparently is an inhibitor of chlorophyll synthesis (Ashton and Crafts, 1973; Herbicide Handbook, 1974). Pentavalent arsenicals, cacodylate and DSMA, are generally weak inhibitors of metabolic activities (Webb, 1966). Similar to mitochondrial work, isolated chloroplasts may not be able to convert them to more potent trivalent forms. Diquat presumably acts as an electron acceptor of photosystem I and competitively inhibits PMS-mediated cyclic photophosphorylation (see Ashton and Crafts, 1973; Zweig et al., 1965). It was thus unexpected to observe no effect in our assays. One reasonable explanation for our failure to see any effect of diquat is that diquat may have less affinity to photosystem I than ferricyanide and methylviologen. Obviously, another explanation for lack of effect of not only diquat but the others is that these herbicides may not be taken up by the chloroplasts.

Diuron (or DCMU) is widely employed as an important inhibitor of photosystem II electron transport. Expectedly, it inhibits at low concentrations the $H_2O \longrightarrow$FeCy electron transport (both coupled and uncoupled) and the $H_2O \longrightarrow$PD (or DMQ) electron transport equally (Fig. III-2A). These results clearly indicate that diuron is an inhibitor of photosystem II activity. Ten other herbicides tested inhibited photosystem II electron transport in a manner identical to diuron, but at different concentrations; atrazine, bromacil, karbutylate, and simazine were effective at micromolar or less; 2,4-D and monuron inhibited at a concentration range of 1-10 µM; DNOC and 2,4,5-T acted at 10-50 µM; and chlorpropham and propham were inhibitory above 100 µM (Fig. III-2, Tables III-1 and III-2). These titer values generally agree with those reported in the literature (see Ashton and Crafts, 1973; Corbett, 1974; Izawa and Good, 1972; Moreland, 1967). The ability of diuron and other herbicides to inhibit photosystem II in turn reflects the phytotoxic potential of these compounds in a mature, intact leaves. Oxygen evolution derived from photolysis of water is a necessary prerequisite for the generation of the reducing power (NADPH) and energy (ATP) needed for reduction of carbon dioxide to carbohydrate in the plant.

No herbicide tested inhibited photosystem I activity. However, diuron and DNOC acted to uncouple this activity at about 1,000 fold higher concentrations than those needed to inhibit photosystem II activity (Fig. III-3). These results confirm previously published data on diuron and nitrophenols (Izawa and Good, 1972; Neuman and Drechsler, 1967).

Titration curves for trifluralin and diallate effects in our standard oxygen electrode assays were different from the diuron type of inhibition. In order to further characterize their effects, phosphorylation studies were carried out with these herbicides (Figs. III-6, 7, 8). Table III-3 summarizes inhibitory effects of these herbicides on chloroplast processes. In this table, additional data of their action on activities of light-stimulated Mg^{++}-ATPase and ATP-Pi exchange reactions are also included. Both herbicides are equally effective in blocking non-cyclic electron transport in coupled chloroplasts, with I_{50} values of 10-20 µM. The diallate inhibition is partially reversed by the addition of an uncoupler (methylamine), evidenced by increase in I_{50} value (Fig. III-5), while uncoupled rate is equally inhibited by trifluralin (Fig. III-4). Diallate is a more effective inhibitor of photosystem II electron transport than trifluralin, but neither herbicide has any effect on photosystem I electron transport. In terms of photophosphorylation, both diallate and trifluralin effectively block non-cyclic phosphorylation; neither inhibits site I phosphorylation at all. Diallate

Table III-3. Summary of inhibitory effects of diallate and trifluralin.

Numbers in parentheses are I_{50} values. + = inhibition; - = no effect.

REACTION	DIALLATE	TRIFLURALIN
ELECTRON TRANSPORT ACTIVITY		
Non-cyclic ($H_2O \longrightarrow FeCy$)	++++ (15 µM)	++++ (10 µM)
Non-cyclic, uncoupled ($H_2O \longrightarrow FeCy$ + methylamine)	+++ (40 µM)	++++ (15 µM)
PS II ($H_2O \longrightarrow PD$, $H_2O \longrightarrow DMQ$)	++ (75 µM)	+ (150 µM)
PS I (Asc/DPIP(or DAD) ⟶ MeV)	-	-
PHOSPHORYLATION ACTIVITY		
Non-cyclic ($H_2O \longrightarrow FeCy$)	++++ (25 µM)	++++ (50 µM)
Site II ($H_2O \longrightarrow DMQ$)	+++ (100 µM)	-
Site I (Asc/DAD ⟶ MeV)	-	-
Cyclic (PMS-mediated)	+++ (60 µM)	-
Light-activated Mg^{++}-ATPase	++ (65 µM)	++ (90 µM)
ATP-Pi exchange	+++ (30 µM)	+++ (25 µM)

blocks site II phosphorylation at high concentrations, while trifluralin has almost no effect (Fig. III-6). Both herbicides inhibit the ATP-Pi exchange reaction rather strongly, while they inhibit the light-activated Mg^{++}-ATPase reaction to a lesser extent. Thus, both these compounds are able to inhibit coupling factor functions which are not directly related to energy conserving electron transport.

Diallate appears to be a unique inhibitor, showing multiple inhibition. It apparently interferes with electron transport between photosystems I and II at a low concentration, while blocking photosystem II electron transfer at higher concentrations. Perhaps, the most unusual diallate effect is the strong inhibition of cyclic phosphorylation, while site I phosphorylation remains untouched. Partial reversal by uncouplers of the electron transfer inhibition suggests a possible role of this herbicide as an energy transfer inhibitor. As for trifluralin, it apparently inhibits primarily the electron transport between the two photosystems, and is thus similar to the water soluble compound EDAC (1-ethyl-3(3-dimethylaminopropyl)-carbodiimide); however, unlike EDAC, it blocks neither cyclic phosphorylation nor photosystem I electron transfer (see McCarty, 1974). Similar to the role played by diuron in the progress of photosynthetic research, trifluralin and diallate may prove to be interesting and important inhibitors of photosynthetic electron transport and phosphorylation.

EVALUATION OF METHOD

Herbicidal phytotoxicity on chloroplast photosynthesis can be expressed in three possible ways: (a) inhibition of photosynthetic electron transport, (b) inhibition of photophosphorylation, and (c) uncoupling of photophosphorylation. If a herbicide acts in any one of the three possible ways, the chloroplasts in plant leaves lose the ability to generate the reducing power (NADPH) and/or energy (ATP) necessary for reduction of carbon dioxide to carbohydrate.

The use of ferricyanide-mediated oxygen evolution by isolated chloroplasts (**Fig.** III-1A) provides a rapid, reliable method for assessing herbicidal phytotoxicity on overall light reactions (both photosystems I and II) of photosynthesis. This method detects all three possible modes of action of herbicides. If the site of herbicidal action must be determined, each photosystem can be examined separately by using appropriate electron donors, mediators, and acceptors. All these methods are simple and reliable using a recording oxygen electrode.

It should, however, be noted that these methods do not distinguish selectivity of a herbicide to act on given groups of plants.

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CHAPTER IV

THE EFFECT OF HERBICIDES ON CHLOROPHYLL FLUORESCENCE IN INTACT SOYBEAN LEAF DISKS

By Charles F. Yocum and Howard H. Robinson

INTRODUCTION

In order to measure photosynthetic activity in whole plants, one must assay either evolution of oxygen or uptake of carbon dioxide. These assays are complicated by the presence of competing reactions (oxygen consumption and carbon dioxide evolution) arising from mitochondrial activity in the same tissue, as well as by the phenomenon of photorespiration. Therefore, one is forced to employ a variety of corrections and complicated experimental approaches to assess photosynthetic activity. We have sought an alternate means to assay phytotoxicity of herbicides on leaf photosynthesis directly. This section of the report details our attempts to employ chlorophyll variable fluorescence as an assay for herbicide phytotoxicity.

Butler (1965) has reviewed the origins of chlorophyll fluorescence in chloroplasts and its relationship to photosynthetic electron transport. The excitation of chlorophyll by light produces a transient state with an unpaired electron in a higher energy level. This is the singlet excited state. There are two possible fates for this electron, one a decay to a new energy level (the triplet state), the other a decay to the original state (ground state). In this latter process energy is lost as light emission or fluorescence. In the former process, triplet state chlorophyll may react with an acceptor molecule (plastoquinone, for example) and retrieve the lost electron from a donor system such as water. Experimental correlations of electron transport (via the triplet state) with fluorescence (from singlet decay) have shown that, under conditions which favor electron transport, the level of chlorophyll fluorescence is at a low level owing to conditions which favor formation of the triplet (reactive) state. If, however, one inhibits electron transport, an increase in the level of chlorophyll fluorescence, denoted variable fluorescence, is observed. A number of studies have shown that the origin of this fluorescence is from the bulk chlorophyll pigments of photosystem II. Diuron. a potent inhibitor of photosystem II (see CHAPTER III), produces high levels of fluorescence when added to chloroplasts.

Variable fluorescence has been used in both chloroplasts and intact organisms to assess the efficiency of photosynthesis. Arnon (1966) has reported that addition of ferredoxin, a natural photosynthetic electron acceptor results in a quenching of chlorophyll fluorescence. Additions of ADP and inorganic phosphate, substrates for photophosphorylation, produced a further decrease in fluorescence. NADP also quenched fluorescence when added along with ferredoxin. Thus a number of naturally occurring cofactors influence the level of fluorescence in isolated chloroplasts. Zweig et al. (1963)

and Zweig and Greenberg (1962) have used measurements of fluorescence to assess the phytotoxic effect of s-triazines, substituted phenylureas, and acylanilides using the unicellular green alga _Chlorella_ for bioassay. These investigators noted an increase in fluorescence simultaneous with a decrease in oxygen evolving activity when herbicides were added to suspensions of these cells.

Since chlorophyll fluorescence is a sensitive indicator of photosynthetic activity which can be readily measured in intact tissue, we have undertaken a series of exploratory experiments to see whether phytotoxicity can be observed in intact higher plant tissues by this procedure. In these experiments, we have relied on our work with isolated chloroplasts (CHAPTER III) to serve as a guide to the phytotoxic effects of the herbicides to be examined. Our preliminary results, reported here, suggest that measurements of chlorophyll fluorescence may provide a rapid, reliable assay for herbicide phytotoxicity in intact plant leaves.

MATERIALS AND METHODS

Construction of the Fluorimeter and Flow Cell. A simple fluorimeter set-up (schematically shown in Fig. 1) was constructed using a Dumont red-sensitive photomultiplier tube as the fluorescence detector. High voltage was supplied by a Hewlett-Packard regulated high voltage power supply, and the output of the tube was amplified by a Keithley 610B electrometer

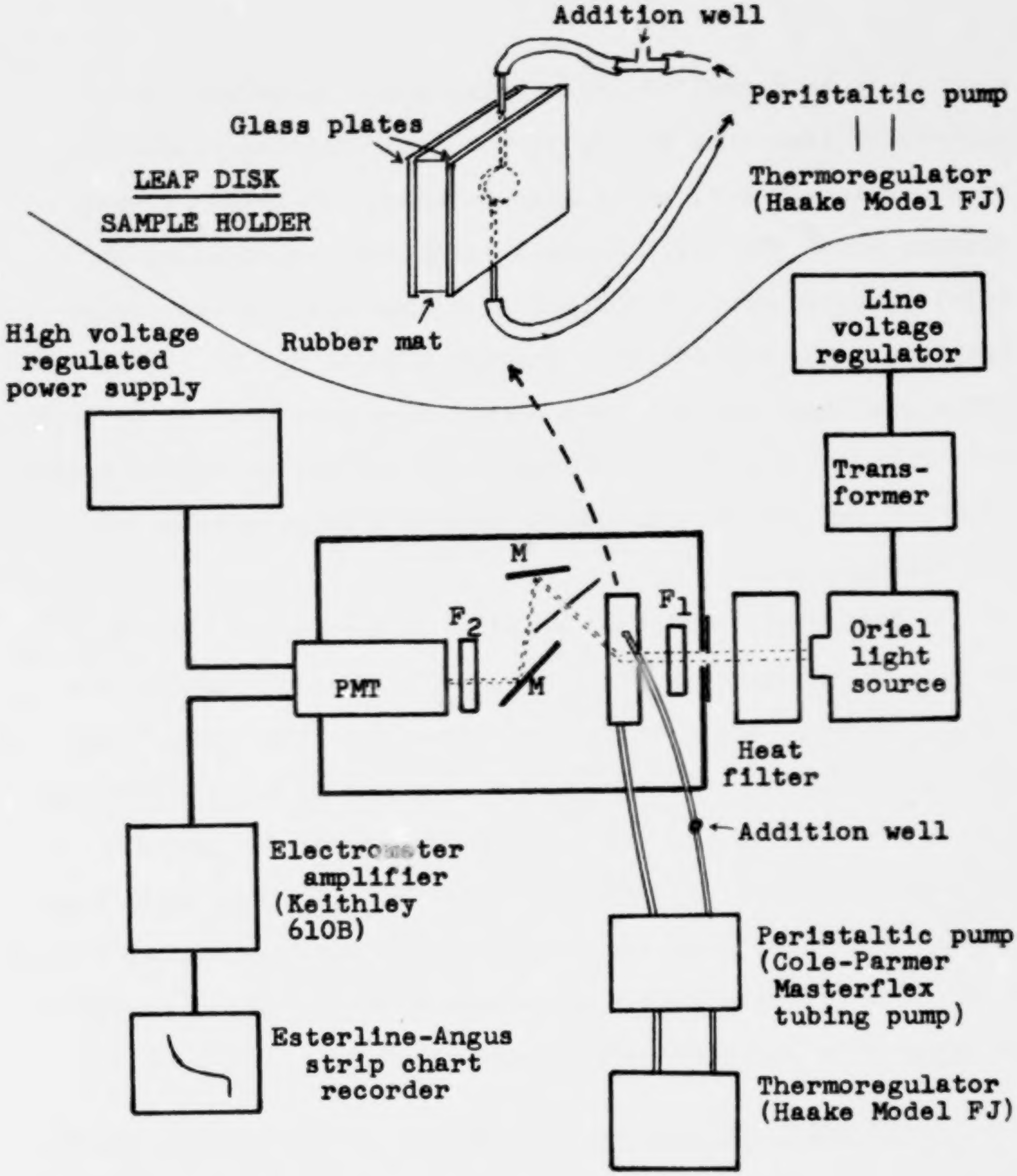

Addition well
Peristaltic pump
Thermoregulator
(Haake Model FJ)
Glass plates
LEAF DISK
SAMPLE HOLDER
Rubber mat
High voltage
regulated
power supply
Line
voltage
regulator
Trans-
former
M
F_2
F_1
Oriel
light
source
PMT
M
Heat
filter
Addition well
Electrometer
amplifier
(Keithley
610B)
Peristaltic pump
(Cole-Parmer
Masterflex
tubing pump)
Esterline-Angus
strip chart
recorder
Thermoregulator
(Haake Model FJ)

Fig. IV-1. Schematic diagram of leaf disk fluorimeter. Top figure details the leaf disk sample holder; leaf sample is place in the center hole. Abbreviations: F_1=primary filter (Corning CS4-96 blue filter); F_2=secondary filter (Corning CS2-64); M=mirror; PMT=photomultiplier tube (Dumont red sensitive).

and fed to an Esterline-Angus strip chart recorder. The photomultiplier tube was protected from exciting radiation by a Corning CS 2-64 red filter (a sharp cut filter transmitting above 590 nm). Exciting light was provided by an Oriel light source, stabilized by a line voltage regulator. Exciting light was filtered through 6 cm of H_2O to remove infra-red light and was then passed through a Corning CS 4-96 blue filter (transmission between 330 and 650 nm with a broad peak between 400 and 480 nm) to remove a large portion of red exciting light.

A leaf-disk sample holder was constructed by cutting a hole, 1 cm in diameter, in a piece of rubber matting (5 x 2.5 x 0.3 cm). Small holes were bored into the center hole from both top and bottom of the rubber mat to admit capillary tubes. The rubber mat was sandwiched between two glass plates with a leaf disk in place and the capillary tubes were connected to a peristaltic pump (Cole-Parmer Master-flex tubing pump). The flowing liquid was temperature regulated at 26° C by means of a Haake Model FJ thermoregulator.

Preparation of Soybean Leaf Disks. Young soybean plants, 10 to 15 day old, were pretreated with a cycle of 12.5 hours of darkness and $\frac{1}{2}$ hour of low intensity light ($\leq$ 1,000 ergs$\cdot$cm$^{-2}\cdot$second^{-1}) prior to excision of leaf disks. This pretreatment optimized fluorescence characteristics of leaf disks. From the first or second fully expanded trifoliate leaf, discks were removed by means of a cork borer and placed in the flow cell of the fluorimeter. The same leaflet was used for both control with diuron and experimental run with a herbicide. The peristaltic pump was activated and water was pumped over the surface of the disk. When the resting level of fluorescence was attained, a herbicide was injected into the system through the addition well and the subsequent effect of this treatment was noted by observing the level of fluorescence on the strip chart recorder.

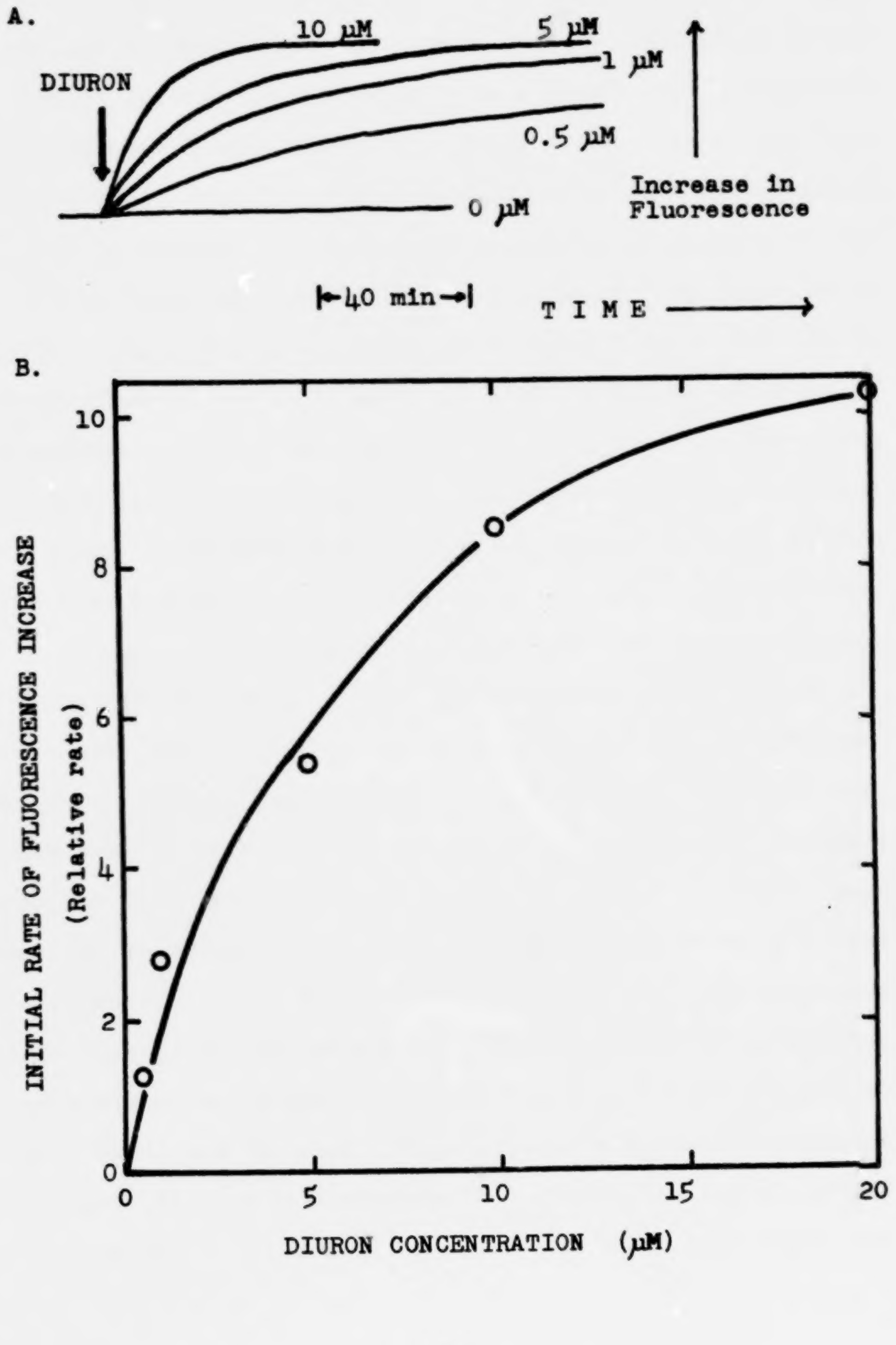
RESULTS

Figure IV-2A illustrates expected increase in fluorescence after addition of diuron to leaf disks. The rate of fluorescence increase is a function of diuron concentrations added. If the initial rise in fluorescence is plotted as the function of diuron concentrations, the results (Fig. IV-2B) indicate that the half-maximal effect is about 4 µM. Since the I_{50} value for photosystem II inhibition in isolated chloroplasts is about 0.1 µM (Fig. III-2A, Tables III-1 and III-2), the half-maximal effect of 4 µM in chlorophyll fluorescence of leaf disks is probably due to slow diffusion of diuron through the leaf into chloroplasts. This contention is further supported by the observation that the final level of fluorescence reached after a long exposure to diuron is about the same for concentrations 1 through 10 µM. It should be mentioned that for each concentration a new leaf disk had to be used; this type of experiments has to be carried out with leaf disks of comparable chlorophyll content.

Fig. IV-2. Effect of diuron concentrations on chlorophyll fluorescence in soybean leaf disks.

A, Recorder traces showing fluorescence increase after treatment with 4 different concentrations of diuron.

B, Initial rate of fluorescence increase as the function of diuron concentrations.


A.
10 µM
5 µM
1 µM
0.5 µM
0 µM
DIURON
Increase in Fluorescence
|←40 min→|
T I M E →
B.
INITIAL RATE OF FLUORESCENCE INCREASE
(Relative rate)
0
2
4
6
8
10
0
5
10
15
20
DIURON CONCENTRATION (µM)

Fluorescence increase patterns were analysed with a number of other herbicides in the manner identical to the diuron experiments. Some herbicides significantly increased chlorophyll fluorescence very slowly (over several hours) over the initial basal level. However, slow fluorescence changes were also noted in the absence of herbicide, presumably reflecting physiological (endogenous) changes of leaf disks under the experimental conditions. As noted in Fig. IV-2A, the final fluorescence attained is not a good criterion, because the analysis takes too long time and it must incorporate possible leaf disk to leaf disk variations. Analyses of initial rate of fluorescence rise include the involvement of diffusion of herbicide and must be standardized. These problems were carefully evaluated in establishment of assay conditions and pretreatment of soybean plants.

Table IV-1 summarizes the results obtained with nine herbicides. In these experiments, two leaf disks were excised from the same leaflet of a trifoliate leaf for each run; one disk was taken from the right side of the mid-vein and the other from the left side of the mid-vein. One disk was treated with a given herbicide and the other with diuron at the same concentration, and the result was expressed on the basis of percentage of diuron effect. At the herbicide concentration of 200 uM, only diuron, bromacil, and atrazine produced an increase in variable fluorescence. None of the other herbicides tested showed an effect on fluorescence rise. If, however, the concentration of herbicide is increased to 400 uM, cacody-

Table IV-1. Effect of nine herbicides on fluorescence increase in isolated soybean leaf disks. Fluorescence increase is expressed as the percentage of the diuron-induced rate at the same concentration.

HERBICIDE	% Rate of increase with:	
	200 µM	400 µM
Diuron (control)	100	100
Amitrole	0	0
Atrazine	80	41
Bromacil	10	11
Cacodylate	0	11
Diquat	0	0
DNOC	0	14
DSMA	0	0
Chlorpropham	0	0

late and DNOC also showed a small phytotoxic response, but DSMA, amitrole, diquat, and chlorpropham did not.

Comparison of these data with soybean chloroplast results shows that amitrole, diquat, and DSMA do not affect either assay. Bromacil, a potent inhibitor of oxygen evolution in isolated chloroplasts (Fig. III-2B, Tables III-1 and III-2), is a weak inhibitor in the fluorescence assay of leaf disks. Chlorpropham, a relatively weak inhibitor of the Hill reaction (I_{50} = 150-250 µM), does not increase leaf fluorescence. It is of interest to note that cacodylate, which shows a small, but significant phytotoxic response in the fluorescence assay, was not inhibitory in the isolated chloroplast assay at all. Thus, in these initial experiments, a reasonable correlation between inhibition of electron transport activity and induction of variable fluorescence was observed, but some herbicides did not show expected responses.

DISCUSSION

These preliminary experiments provide evidence for the usefulness of chlorophyll fluorescence as a tool to assess phytotoxicity of herbicides. In the experiments reported here, we have observed a phytotoxic effect with 6 out of 9 herbicides tested. Furthermore, we have a preliminary indication that in one case, cacodylate, the fluorescence assay reveals phytotoxicity, whereas the organelle assay did not. This herbicide may act on a system closely associated with the light reactions of photosynthesis either directly or after metabolic conversion into an active form. Thus, the advantage of this fluorescence method is that it extends the sensitivity of the assay to intact tissue, and provides the capability of assessing the toxic effect of herbicides whose primary sites of inhibition are not in the photosynthetic electron transport chain.

In these preliminary studies, we have sought only to demonstrate that fluorescence measurements can serve as a means of detecting phytotoxic responses to certain herbicides. Before this method can be reliably applied to all herbicides, further work must be done to refine the technique. There are a number of problems which must be resolved.

We have used leaf tissue with buffered solutions in a flow system. While this condition did not affect fluorescence in the periods of time we have used for assay, this is obviously not an ideal system since it bears no relationship to the natural system for herbicide application. We must ultimately find a means to apply herbicides to an intact leaf in the ordinary atmosphere and observe the resulting effect on chlorophyll fluorescence.

Furthermore, we have examined the effect of herbicides on fluorescence for short periods of time (30-60 minutes). While these times may be adequate for fast-acting inhibitors, they do not allow for assay of slower-acting herbicides which may be equally inhibitory over a longer period of time. We are currently restricted to these short assay times because our illumination arrangement (blue light excitation) causes a gradual increase in tissue fluorescence. In order to solve this problem we must investigate the use of various filter arrangements to see whether we can stabilize intact tissue at low levels of fluorescence for longer periods of time (perhaps days) and subsequently eliciting a response upon herbicide addition.

EVALUATION OF METHOD

Chlorophyll fluorescence can provide a test for herbicide toxicity under the conditions reported here. If, however, it is to serve as a reliable test for phytotoxicity for plants under natural conditions, several problems must still be solved.

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CHAPTER V

HERBICIDAL EFFECTS ON CORN ROOT TIP RESPIRATION AND SOYBEAN LEAF DISK PHOTOSYNTHESIS

By H. Ikuma, S. Kisler, and W. R. Taylor

INTRODUCTION

Studies with isolated organelles can provide biochemical modes of action of herbicide phytotoxicity relatively readily (see CHAPTERS II and III), but they fail to detect the selectivity of herbicide action on different types of plants and on the same plants in different physiological conditions (pre-emergence, post-emergence, etc.) (see Ashton and Crafts, 1973). Herbicides applied in the field must be taken up by the plants in the original forms or in modified forms (by bacterial action in the soil) before they become effective in inhibiting plant metabolism. They may be further modified in the plant before eliciting their effects or they may be detoxified in the resistant plants. In the studies with isolated organelles, we could not observe possible *in vivo* action of herbicides. In our preliminary sets of experiments on chlorophyll fluorescence with isolated soybean disks, we have already witnessed some discrepancies in results between isolated organelle studies and leaf fluorescence characteristics (CHAPTER IV). In general, isolated organelles tend to exhibit practically identical responses, regardless of their sources, to inhibitors such as herbicides (CHAPTERS II and III). In order to circumvent these problems and to examine herbicidal action more closely to intact plants, the investigations in this section was undertaken with isolated tissues. Tissue selected for these investigations were corn root tips for tissue respiration and soybean leaf disks for photosynthesis; these tissues were selected to compare the results with other projects in this report.

Respiration and photosynthesis are two important processes of plants which herbicides may exert phytotoxic action (Ashton and Crafts, 1973; Moreland, 1967). Two gases (oxygen and carbon dioxide) are involved in these processes; oxygen is consumed and carbon dioxide evolved in respiration, but photosynthesis consumes carbon dioxide and evolves oxygen. In both respiration and photosynthesis, oxygen-involved pathways are different from carbon dioxide-involved pathways. In our organelle studies (CHAPTERS II and III), our attention has been given to the oxygen-involved processes. In the investigations with plant tissues, we considered it highly important to analyze overall reactions of respiration or photosynthesis; i.e., both oxygen- and carbon dioxide-involved metabolic steps must be examined in either respiratory or photosynthetic study. For this purpose, either study was carried out by simultaneous measurements of oxygen (with an oxygen electrode) and carbon dioxide (with a carbon dioxide electrode).

MATERIALS AND METHODS

Respiration of Corn Root Tips. From 3 day old, dark grown corn (Zea mays Michigan Hybrid 200) seedlings, 3-mm primary root tips were excised by means of a home-made cutting device. Root tips (50-100) were immersed in 5% (v/v) commercial Clorox solution, followed by rinsing three times with excess amount of sterilized distilled water. The root tips were then introduced to a lucite cuvette of 5.5 or 4.8 ml capacity, which contained 10 mM potassium phosphate buffer at pH 7.0 and 50 mM sucrose. The lucite cuvette housed an oxygen electrode (No. 4004, Yellow Springs Instrument Co., Yellow Springs, Ohio) and a carbon dioxide electrode (E5037, London Co., Cleveland, Ohio). The oxygen electrode was connected to a Heath-Schlumberger recorder (SR-255-B) via a home-made circuit box as in our studies with isolated organelles. The carbon dioxide electrode was connected to a Radiometer pH meter (PHM 71, Mk 2, London Co.) with a CO_2 module (PHA 935, London Co.), the output of which was connected to a Model SR-255-B Heath-Schlumberger recorder. The cuvette was placed above a magnetic stirrer and the content was stirred at a speed not to disrupt root tips, but to allow the uniform dispersal of dissolved oxygen in the cuvette.

Both oxygen consumption and carbon dioxide evolution was examined with electrodes for 20 to 25 minutes. The cuvette was bubbled with air for 5 to 10 minutes to keep the root tips aerated and stirred. Thirty minutes after the start of the first measurements of respiration, respiratory rates were again measured for 20 to 25 minutes, and the cuvette was aerated again. Respiratory measurements were successively made every 30 minutes for the first 1½ hours to obtain the rates prior to herbicide addition. Immediately before the fourth run, a herbicide was introduced to the cuvette, and respiration was measured. Usually herbicide does not affect respiratory rate immediately after addition. About 2 hours after addition, therefore, respiratory rates were examined to analyze herbicidal effects on respiration.

Photosynthesis by soybean leaf disks. From the first or second trifoliate leaf of a young soybean plant (about 3 weeks old, grown in the Mattaei Botanical Gardens of the University of Michigan), 10 leaf disks, each 7 mm in diameter, were made with a No. 3 cork borer. They were vacuum-infiltrated with a herbicide solution or distilled water containing about 1% or less concentration of DMSO, dark-incubated in a solution composed of 10 mM potassium phosphate buffer at pH 7.0, 4 mM $KHCO_3$, and a herbicide for about one hour, and positioned with an insect pin in the same cuvette as that used for corn root respiration. Vacuum infiltration was performed to increase the uptake of herbicide by air-space filled leaf disks and thus to make quick measurements of herbicidal effects. The rate of dark respiration was first examined with an oxygen and a carbon dioxide electrodes for about 15 minutes, then saturating intensity of light (about 10^7 ergs per cm^2 per second) was given through a water, heat filter

(6 cm) to measure both oxygen evolution and carbon dioxide uptake. After measurements, chlorophyll was extracted with 80% ethanol by the method of Arnon (1949), and the net rate of photosynthesis (apparent photosynthesis rate minus dark respiration rate) was expressed on the basis of unit chlorophyll content.

RESULTS

Respiration of Corn Root Tips. A typical set of eight consecutive traces of oxygen and carbon dioxide, each started every 30 minutes, is shown in Fig. V-1. There was no herbicide given in this run. Oxygen electrode traces (Fig. V-1A) are curving, indicating possible diffusional limitation of oxygen into the root tips. On the other hand, traces of carbon dioxide (Fig. V-1B) show delayed response, signifying a much slower response time of the carbon dioxide electrode than that of the oxygen electrode. Many attempts were made to improve the performance of carbon dioxide electrode, but we were unable to do so. Because of these problems, the rate of oxygen uptake was calculated from the initial linear portion of the trace and the rate of carbon dioxide evolution from the later linear portion. An average rate of oxygen uptake with 6 different batches of root tips was 570 nmole oxygen consumed/minute·g fresh weight (standard error = 46) and that of carbon dioxide evolution with 7 determinations was 605 nmoles CO_2 evolved/minute·g fresh weight

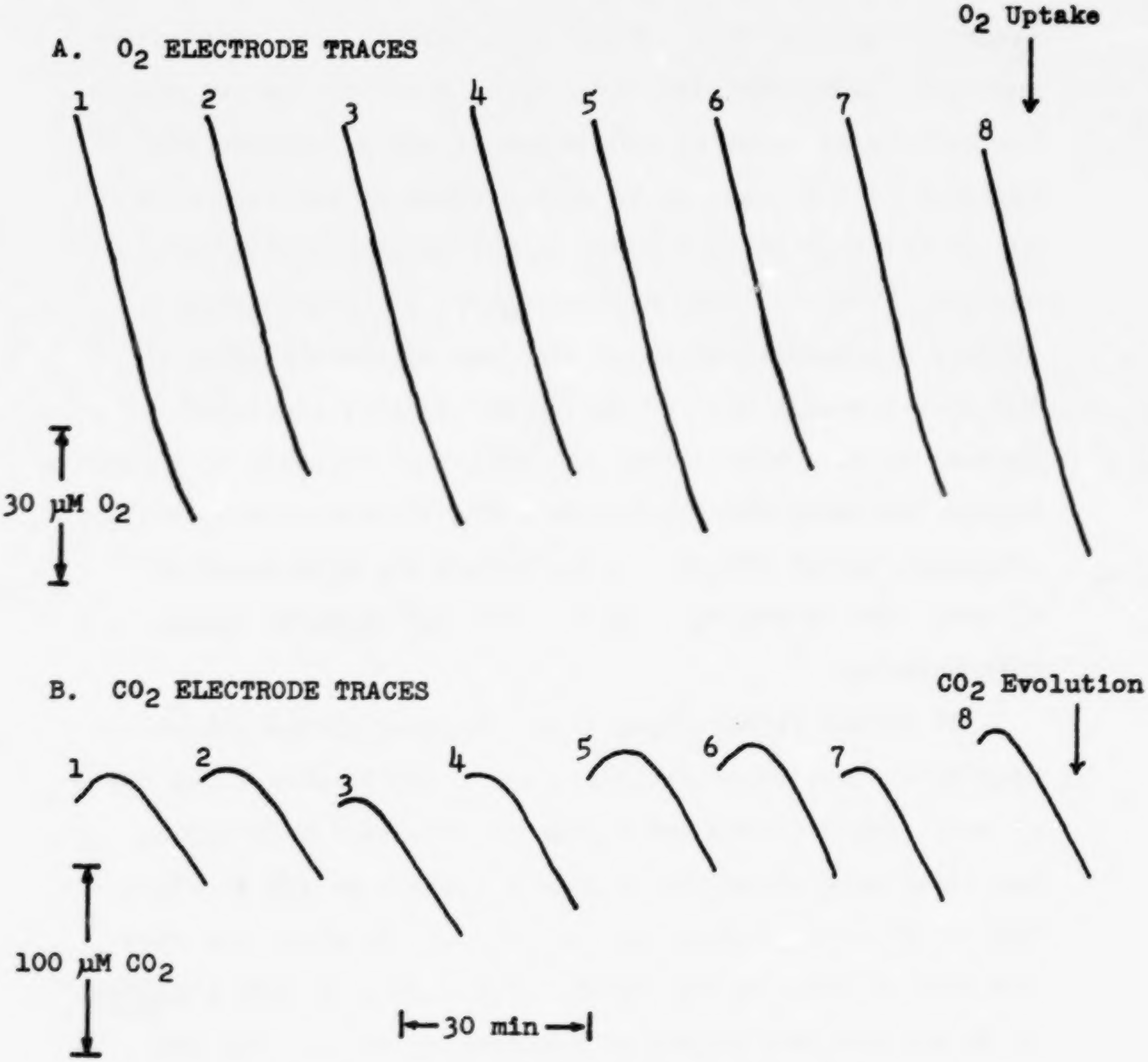

Fig. V-1. A set of recorder traces of oxygen (A) and carbon dioxide (B) elctrodes, showing respiratory activity of corn root tips.

The numbers on the traces indicate successive runs (every 30 minutes) with the same control root tips without herbicide treatment. No. 1 traces were made immediately after placing the tips into the measuring cuvette.

(standard error = 130). From these results, the respiratory quotient (carbon dioxide rate/oxygen rate) can be calculated; the calculated value of 1.06 which strongly suggests the respiratory substrate to be carbohydrate is expected from the addition of 50 mM sucrose in the bathing medium (cf. Beevers, 1961). A high standard error value of carbon dioxide determination (about 20%) may be associated with the slow response time of the carbon dioxide electrode; because of this high value, the root tips response to herbicide beyond 20% level was considered a significant effect, while responses within 20% of control values are considered no effect. The confidence limit of 10% was used for oxygen measurements.

It should be mentioned that DMSO used as the solvent of many water insoluble herbicides was found to change the rates of both oxygen uptake and carbon dioxide evolution during the first hour after DMSO addition (less than 10% in final concentration). During the second hour, however, the rate returned to the original level. These observations prompted us to use the measurement of respiratory rates after two hours from the herbicide addition. Respiratory measurements after two to three hours from herbicide or DMSO addition were more variable; some batches gradually declined the respiratory activity, while others maintained the earlier activity. This increased batch to batch variation was also considered in deciding the measurement period to the two hour after herbicide or DMSO addition.

Using the concentration range of 10^{-6} to 10^{-3} M, the effects of all 17 herbicides were examined. Only three herbicides (2,4-D, DNOC, and 2,4,5-T) caused slight effects at high concentrations (Table V-1). DNOC inhibited respiration slightly at 10^{-3} M, while it stimulated at 10^{-4} M. This DNOC effect is anticipated from the mitochondrial work that this herbicide is a potent uncoupler of oxidative phosphorylation of isolated mitochondria at 10^{-4} to 10^{-5} M and an inhibitor of electron transport at 10^{-3} M (CHAPTER II). However, the effects were small in magnitude. At 10^{-3} M, 2,4-D and 2,4,5-T slightly inhibited oxygen uptake, but carbon dioxide evolution was either slightly stimulated or not affected. This differential effect is of interest, but the magnitude of the effects was small and the effective concentration was high. In addition, in view of drastic effects these herbicides cause in the ultrastructure of root tip cells (CHAPTER VII) at this concentration, the differential effect which was repeatedly observed at 10^{-3} M was not further examined. All other herbicides caused no effect even at 10^{-3} M.

Photosynthesis by Soybean Leaf Disks. When water-infiltrated leaf disks were alternately exposed to darkness and light, their photosynthetic activity, but not their respiratory activity, was found to decrease with time. Attempts were thus made to alleviate this difficulty; leaf disks were treated with various concentrations of bicarbonate, osmotica, light intensities, lengths of darkness and light,

Table V-1. Effects of three effective herbicides on respiratory activity of isolated corn root tips. The results are expressed in terms of percentages of control. Control values are 570 nmoles/min·g fresh weight for oxygen uptake and 605 nmoles/min·g fresh weight for carbon dioxide evolution. Errors of measurements were 10% for oxygen uptake and 20% for carbon dioxide evolution.

HERBICIDE	Concentration used	Effect on O_2 Uptake	Effect on CO_2 Evolution
	(M)	(% of control)	
2,4-D	10^{-3}	85	126
	10^{-4}	93	106
DNOC	10^{-3}	67	79
	10^{-4}	121	150
	10^{-5}	101	108
2,4,5-T	10^{-3}	88	102
	10^{-4}	101	110

and various degrees of washing. We also examined a possible use of shredded leaf instead of disks. The major problem turned out to be light; once the infiltrated leaf disks (or shredded leaf) were treated with light, subsequent light treatments after intervening dark periods were incapable of restoring full photosynthetic capability in the disks, and the rate of photosynthesis decreased with increasing number of dark-light cycles. If the discs were infiltrated in weak diffuse light and incubated in the dark for one hour, the rate of photosynthesis in subsequent light was practically identical to that measured immediately after infiltration. Based on these preliminary sets of experiments, we have adopted the procedure described in MATERIALS AND METHODS.

With this method, however, herbicidal effects are not examined with the same leaf disks used for control measurements. Control disks must be examined simultaneously with the experiments with herbicides. We have, therefore, used the disks prepared from the same trifoliate leaves for both control and experimental runs. Even with this precautions, experimental variations increased over 30%. For example, an average of 11 runs for net oxygen evolution studies was 0.778 nmoles/minute·ug chlorophyll with a standard error of 0.23 nmoles/minute·ug chlorophyll; and an average of 10 runs for net carbon dioxide uptake was 0.579 nmoles with a standard error of 0.15 nmoles on the same unit as above. As noted in the average values, the ratio of oxygen evolved to carbon dioxide taken up (1.34 in these measurements) deviates significantly from the expected value of 1.0. We ascribe this deviation to the performance of carbon dioxide electrode. These preliminary observations forced us to examine herbicidal effects more qualitatively than we expected.

Similar to root tip respiration work, the effect of each herbicide was examined for concentrations from 10^{-6} to 10^{-3} M. Table V-2 shows the effects of all 17 herbicides on net photosynthesis as percentages of control values. It should be added that these herbicides affected leaf disk respiration very little.

Of the 17 herbicides tested, eight herbicides (amitrole, cacodylate, chlorpropham, 2,4-D, DSMA, propham, 2,4,5-T, and trifluralin) exerted very little effect at concentrations of 10^{-4} and 10^{-3} M. Bromacil was by far the most potent herbicide inhibiting practically completely at 10^{-6} M. Other eight herbicides (atrazine, diallate, diquat, diuron, DNOC, karbutylate, monuron, and simazine) caused strong inhibition at the concentration range of 10^{-5} to 10^{-4} M. Most of these effects parallel the results with isolated chloroplasts (Table III-2). Note, however, that with an exception of bromacil effective herbicides inhibited photosynthesis at higher concentrations with leaf disks than with isolated chloroplasts. This signifies that the uptake and/or metabolism of herbicides must play a role in phytotoxicity. Although measured rates of oxygen evolution differed from those of carbon dioxide uptake significantly (see above), the results expressed in terms of per cent of control agree well between the oxygen electrode and the carbon dioxide electrode readings (Table V-2). Perhaps, the treatment with diquat is the only herbicide which showed consistently lower carbon dioxide uptake than oxygen evolution; the results agree with the notion that this herbicide acts as an electron acceptor of photosystem I (Ashton and Crafts, 1973).

DISCUSSION

Studies with isolated tissues are in general more variable in response to stimuli than uniformly suspended organelles. Variability can be caused by slight differences among plants which have presumably been grown under the uniform conditions. In order to circumvet such a problem, an ideal testing method is to use the same batch of tissues for both control and experimental treatments. This can be accomplished by treating the tissue before undergoing physiological changes and compare the results before and after the treatment. We could perform such a method with isolated corn root tips, but not with soybean leaf disks. Even in our study with isolated root tips, we experienced relatively high experimental variations of 20%. When control and experimental treatments were separately run as in the leaf disk photosynthesis work, the experimental errors increased over 30%.

Another source of experimental error exists in the techniques of measurement. Carbon dioxide measurements are highly important in the type of study we undertook, but the

Table V-2. Effects of 17 herbicides on photosynthesis of isolated soybean leaf disks. The results are expressed in terms of % of control; experimental errors were unfortunately large (about 35%) for the measurements of oxygen evolution and carbon dioxide uptake.

HERBICIDE	Concentration used (M)	Effect on	
		O_2 Evolution	CO_2 Uptake
		(% of control)	
Amitrole	10^{-3}	71	55
Atrazine	10^{-6}	93	88
	10^{-5}	19	0
	10^{-4}	5	0
Bromacil	10^{-6}	8	0
	10^{-5}	9	0
Cacodylate	10^{-3}	112	112
Chlorpropham	10^{-3}	72	76
2,4-D	10^{-3}	119	114
Diallate	10^{-5}	115	96
	10^{-4}	0	0
Diquat	10^{-6}	100	40
	10^{-5}	64	0
	10^{-4}	39	0
	10^{-3}	0	0
Diuron	10^{-6}	55	57
	10^{-5}	20	9
	10^{-4}	0	0
DNOC	10^{-5}	100	76
	10^{-4}	19	20
	10^{-3}	0	0
DSMA	10^{-3}	114	67
Karbutylate	10^{-6}	81	57
	10^{-5}	39	0
	10^{-4}	0	0
Monuron	10^{-6}	109	54
	10^{-5}	5	27
	10^{-4}	7	7
Propham	10^{-4}	128	133
	10^{-3}	52	55
Simazine	10^{-6}	90	104
	10^{-5}	11	1
	10^{-4}	0	0
2,4,5-T	10^{-4}	137	93
	10^{-3}	69	16
Trifluralin	10^{-4}	77	64

electrode we employed is not as stable as we initially expected (see Severinghaus and Bradley, 1971). Long response times and instability of the electrode were the major problems. The oxygen electrode, on the other hand, was more reliable with a faster response time. Handling of tissues may not have been optimal in our experiments; root tips underwent noticeable change in respiratory activity after a few hours in the cuvette with intermittent aeration, and vacuum infiltrated soybean leaf disks showed decreasing photosynthetic activity more than one light-dark cycles. Ideally, root respiration must be measured in the soil conditions and leaf photosynthesis under normal atmospheric conditions. Despite these technical problems, the results obtained showed good agreement with those obtained with isolated organelles.

Importance of simultaneous measurements of oxygen and carbon dioxide in the study of tissue respiration and photosynthesis is seen from a theoretical ground that the oxygen-involved process and carbon dioxide-involved process are different in cellular metabolism (INTRODUCTION). This importance is in part shown in the differential effect of diquat on leaf disk photosynthesis (Table V-2). In general, however, the results from tissue studies agreed the results with isolated organelles which was carried out with the oxygen electrode alone. This agreement can be taken to indicate that primary modes of action of herbicides in inhibition of respiration and photosynthesis in tissues are on the electron transport chain activity.

EVALUATION OF METHOD

Both respiration and photosynthesis involves the same exchanging gases (oxygen and carbon dioxide). The process which oxygen is directly involved (electron transport) is different from, but linked with, the process where carbon dioxide is involved (Krebs cycle or Calvin cycle). Phytotoxicity of herbicides in plant respiration and photosynthesis may exhibit itself in one or more of the following four ways:

(1) A herbicide acts to inhibit electron transport and/or associated phosphorylative activity, thereby inhibiting the carbon dioxide-involved process as well;

(2) A herbicide acts to uncouple phosphorylation from electron transport, thereby stimulating the activity of both electron transport and the carbon dioxide-involved process in the case of respiration,or stimulating the electron transport activity,but inhibiting the carbon dioxide-involved process,in the case of photosynthesis;

(3) A herbicide acts to inhibit the carbon dioxide-involved process and consequently causes decreases in the oxygen-involved process; and

(4) A herbicide acts to inhibit the energy metabolism of the cell, thus slowing both oxygen- and carbon dioxide-involved processes.

Simultaneous measurement of both oxygen and carbon dioxide thus becomes highly important in assessing possible phytotoxicity of herbicides, particularly when intact cells or tissues are employed.

However, technical, biological, and chemical problems which arose during the course of the present study cast doubts as to the desirability of the simultaneous measurement. The problems were as follows:

(a) the carbon dioxide electrode used did not perform to the same reliability as the oxygen electrode;

(b) isolated tissues, particularly leaf disks, showed relatively high experimental variations and also time-dependent physiological changes; and

(c) all herbicides tested exhibited the effects which can be readily interpreted with the use of an oxygen electrode alone.

The first problem can be solved by the development or the use of a more reliable carbon dioxide electrode. We did not explore all commercially available electrodes. In order to circumvent the second problem, we propose the use of intact plants, rather than isolated tissues, grown hydroponically and to measure root respiration in the solution and shoot photosynthesis via an appropriate method. Excision of tissues from plants and subsequent treatment (e.g., Clorox treatment of root tips and vacuum infiltration of leaf disks) must be responsible in causing physiological changes in the excised tissues. We expect hydroponically-grown plants to show more consistent and reliable patterns of gas exchange and to respond to the application of a herbicide in a manner similar to the plants in the field. The third point may be coincidental and depends upon the type of herbicides tested.

Although much effort was given in this study to solve problems, simultaneous measurement of two exchanging gases will be of great use in assessing phytotoxicity of herbicides. In addition, the establishment of method of simultaneous measurement will enable us to examine selectivity of herbicides and also to eliminate organelle study.

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CHAPTER VI

HERBICIDE EFFECTS ON PROTEIN AND NUCLEIC ACID SYNTHESES

By E. J. Bowman, M. Virmani, and H. Ikuma

INTRODUCTION

Proteins and nucleic acids play very important roles in cellular functions and cellular multiplification (see Giese, 1968). Proteins are composed of amino acids and nucleic acids of nucleotides. Proteins are important in cellular function as parts of structural units (cell wall, various membranes, ribosomes, chromosomes, etc.) and also as enzymes. Four types of nucleic acids exist in the cell; DNA as the source of genetic information, ribosomal RNA (rRNA) providing the site for protein synthesis, transfer RNA (tRNA) as the carriers of amino acids in protein synthesis, and messenger RNA (mRNA) as the messenger of genetic information in DNA for proper sequencing of amino acids into a protein.

According to the current dogma, protein synthesis involves two steps; (1) the transcriptional step in which mRNA is made on DNA, and (2) the translational step in which genetic information transcribed on mRNA is translated into a polypeptide chain on ribosomes. New DNA molecules are made on pre-existing DNA during the cell cycle, and all three types of RNA are also made on DNA template. In actively dividing cells, DNA replication and RNA and protein syntheses take place.

Because of the importance of protein and nucleic acids in cellular function, it is highly likely that some herbicides may cause their phytotoxic action in the machinery of their syntheses (see reviews by Moreland, 1967; Ashton and Crafts, 1973). We chose to investigate phytotoxic action on protein and nucleic acid syntheses using corn root tips as the experimental material. The choice was made because root tip cells are actively dividing and also actively synthesizing proteins. In addition, some methodological and physiological studies had been fairly well carried out (Noodén, 1971, 1972; Ogur and Rosen, 1950; Woodstock and Skoog, 1960). Furthermore, the nature of the root to absorb chemicals from soil solution was considered advantageous in the study of phytotoxicity of herbicides.

The method employed in this study is the incorporation of respective radioactive precursors into DNA, RNA, and proteins (Noodén and Thimann, 1963; Noodén, 1971, 1972; Mann et al., 1965; Moreland et al., 1969). The radioactive precursors were thymidine for DNA, uridine for RNA, and leucine for protein. This frequently employed method is an indirect way of analyzing protein and nucleic acid syntheses, depending upon the uptake and specific incorporation of precursors into respective polymers. Since the precursors can be metabolized into other compounds than the polymers of interest if root tips are incubated for a long time with the precursors, the incubation time for the present work was set at two hours. This short period of incubation did not allow tissue to take up much precursors, and it was also found that the incorporation was not as specific as we expected. In the herbicide literature, this type of study has been reported (Mann et al., 1965; Moreland et al., 1969; Noodén, 1972). These studies were carried out (a) with the use of non-dividing tissues (Mann et al., 1965; Moreland et al., 1969), (b) with no attention given to DNA replication, (c) with long incubation time (Moreland et al., 1969; Noodén, 1972), and/or (d) without critical analysis of method. In this series of research, therefore, we paid particular attention to analyze the assay technique critically as well as to survey herbicidal phytotoxicity in protein and nucleic acid syntheses.

MATERIALS AND METHODS

Preparation of Corn Seedlings. Seeds of Zea mays L. Michigan Hybrid 200 were soaked in 5% (v/v) commercial Clorox solution for 10 minutes followed by washing in running tap water for 2 hours. They were soaked in a large volume of tap water with aeration for 6 to 8 hours, and spread in a single layer on a perforated aluminum sheet which was suspended in a 8" x 12" x 2½" planting tray. Tap water was added to the level of seeds and seeds were germinated at 26° C for 3 days in the dark.

Incubation of Root Tips with Radioactive Precursors of Protein and Nucleic Acids. From the primary roots of corn seedlings (1-3 cm long), 3-mm tips were separated by means of a home-made cutting device. Root tips were surface sterilized by soaking in 5% (v/v) commercial Clorox solution for 5 minutes, followed by thorough rinsing with sterile distilled water with several changes. A group of 10 to 25 root tips were then placed in a small flask or vial with 5 ml or 0.5 ml of the incubation medium containing 50 mM sterilized glucose and herbicide. After 30 minutes, a small amount of ^{14}C-leucine + ^{3}H-thymidine or ^{14}C-uridine was added. The radioisotopes were given at 1 or 2 x 10^6 CPM without carriers. The flask was aerated by means of shaking throughout the incubation period at room temperature (25 ± 1° C). Two hours after the addition of radioisotope(s), root tips were quickly washed with sterilized distilled water and subjected to fractionation of DNA, RNA, and protein.

Separation of DNA, RNA, and Protein Fractions. Initially, the perchloric acid method of Ogur and Rosen (1950), modified by Woodstock and Skoog (1960), was employed. This method depends on differential solubilization of DNA and RNA in acid at different temperatures. Because this method could not separate the three fractions clearly, an enzymatic method was developed using DNase and RNase to solubilize DNA and RNA, respectively. The enzymatic method was further modified to improve the efficiency of analyses of herbicidal effects.

(i) The First Enzymatic Method. Root tips in 5 ml incubation medium were quickly washed with sterile distilled water and subjected to the following four step fractionation procedure:

a. Homogenization step. Root tips were homogenized in 5 ml of cold (0-4° C) 75% ethanol with a motarized Teflon tissue grinder. DNA, RNA, and protein were precipitated and collected by centrifugation. This and all subsequent centrifugation steps were carried out at about 10,000 x g for 15 minutes in a swinging bucket rotor.

b. De-lipidation step. The pellet from the preceding step was successively treated with acidified 75% ethanol (0.1 N perchloric acid in 75% ethanol) at room temperature for a few minutes, ethanol-ether (3 : 1) at 60° C for 5 minutes, and 75% ethanol at 0-4° C. Each treatment involved homogenization of the pellet and centrifugation.

c. Hydrolysis of RNA by RNase and removal of DNA + protein for ^{14}C-uridine incorporation experiment. The pellet from the preceding step was uniformly suspended and incubated in 1 ml of 0.1 M sodium acetate buffer (pH 5.0) containing 2 mg RNase at 37° C for 4 hours. DNA and protein were removed by adding 4 ml of cold ethanol and centrifuging the mixture. The pellet was washed with 2 ml of 75% ethanol. The combined supernatant was used for radioactivity counting.

d. Hydrolysis of DNA by DNase and separation of protein for the ^{14}C-leucine + ^{3}H-thymidine incorporation experiment. The pellet from step b was uniformly suspended in 1 ml of 0.1 M tris-HCl buffer (pH 8.0) containing 0.4 mM $MgCl_2$ and 2 mg DNase at 37° C for 4 hours. The reaction was stopped and protein + RNA precipitated, by addition of 1 ml of 1 N perchloric acid (0.5 N final concentration). The mixture was centrifuged and supernatant was directly counted in a scintillation counter for DNA analysis. The pellet was suspended in 100% ethanol and filtered through a Schleider & Schuell filter (# 595). The filter pad containing protein and RNA was washed again with ethanol and counted in a scintillation counter.

(ii) The Second (Modified) Enzymatic Method.

a. Experiments with ^{14}C-uridine. Root tips in 0.5 ml incubation medium were quickly washed with sterile distilled water and homogenized as above. The resultant pellet was subjected to hydrolysis by RNase as in step c of the first method.

b. Enxperiments with ^{3}H-thymidine + ^{14}C-leucine. Root tips in 0.5 ml incubation medium were quickly washed and homogenized as above. The resultant pellet was subjected to hydrolysis by DNase as in step d of the first method.

The second method was carried out with a reduced volume (0.5 ml) of the incubation medium, and it by-passes the de-lipidation step (step b of the first method). The reduced volume of the incubation medium increases the efficiency of incorporation. The de-lipidation step was found unnecessary for corn root tips.

In some cases, particularly when a single isotope was used, the pellet obtained in the homogenization step was directly employed for radioactivity measurements. This simplified method is particularly applicable for experiments with ^{14}C-leucine alone.

Radioisotope Counting Method. Earlier counting was done with a Packard Tricarb Scintillation counter using the standard scintillation fluid composed of toluene and POPOP. Since the presence of perchloric acid caused as high as 90% quenching, the counting method was changed to a triton method (1 part triton X-100, 2 parts toluene, and 5 mg/l PPO) using a Beckman scintillation counter (Model LS-230) at room temperature. This method showed no detectable quenching in the presence of 1 N perchloric acid.

Chemicals and Equipment. All chemicals were of analytical reagent grade or best commercially available grade. DNase (deoxyribonuclease I, chromatographically prepared) and RNase (ribonuclease A, Type III-A and Type XI-A) were purchased from Sigma Chemical Co. Of the two types of RNase obtained, Type XI-A did not contain protease and suitable for this study, while Type III-A showed an appreciable amount of protease activity. The latter was used in some experiments, because of unavailability of the better enzyme. ^{14}C-Leucine (uniformly labeled, specific activity 283 mc/mM), ^{14}C-uridine (uniformly labeled, specific activity 472 mc/mM), and ^{3}H-thymidine (methyl-H labeled, specific activity 50.8 Ci/mmole) were purchased from New England Nuclear. Most herbicides were dissolved in dimethylsulfoxide (DMSO) and necessary dilutions were made into the incubation medium from the stock solutions. The concentrations of DMSO in the incubation medium less than 1% did not affect the uptake and incorporation patterns of radioactive precursors of polymers, but DMSO at 10% caused marked inhibition of uptake and incorporation. The concentration of DMSO in the incubation medium was set less than 1%; this effectively made us to study the herbicidal effects up to 10^{-4} M.

The motarized tissue grinder was a tissue grinder whose pestle was connected to a Montgomery Ward drill. The teflon tissue grinder was purchased from A. H. Thomas Co. A Sorvall refrigerated RC-5 centrifuge with a swinging bucket rotor (HB-4) was used in centrifugation.

RESULTS

Development of Enzymatic Methods. Since high metabolic and mitotic activities are localized in the tip 1-2 mm region of the corn root (Woodstock and Skoog, 1960), we have decided to employ the 3 mm root tip sections in this study. Corn root tips are surrounded by slimy substance, presumably of polysaccharide in nature, which may contain some bacteria. In order to examine possible involvement of bacteria, root tips were either pretreated with 5% Clorox or incubated with chloramphenicol. Table VI-1 shows the results from a typical series of experiments. Chloramphenicol treatment resulted in 26% less counts and Clorox pretreatment in 43% less counts than the untreated control. In another series of experiments, it was found that a combined treatment with both Clorox and chloramphenicol was as effective as the Clorox treatment alone. The Clorox pretreatment step was thus introduced into our routine procedure.

The two hour incubation period was adopted in these studies to examine both early effects of herbicides and the effects prior to possible metabolism of added precursors of nucleic acids and protein. The incubation medium contained 50 mM glucose to avoid starvation of root tips during treatment. Since radioisotopes were taken up linearly with time, herbicide was added 30 minutes prior to introduction of radioisotopes to secure the uptake of herbicide by the root tips and its effects on protein and nucleic acid syntheses.

Table VI-1. Examination of possible contribution of bacteria in ^{14}C-leucine incorporation into root protein. 10 root tips, treated or untreated, were incubated with about 10^6 CPM ^{14}C-leucine for 2 hours. Root tips were homogenized in 75% ethanol, and the pellet was counted in a scintillation counter. An average of duplicate runs are given in the Table.

Treatment	Radioactivity in Protein	
	(CPM/gm FW)	%
Control (no treatment)	45,000	100
Clorox treatment prior to incubation	26,100	57
Chloramphenicol (10^{-4} M) during incubation	33,100	74

The perchloric acid extraction method of Woodstock and Skoog (1970) is relatively simple but allows some DNA, RNA, and proteins to be hydrolyzed excessively or incompletely. One of the typical set of results is presented in Table VI-2. As evident from the results, only 43% of the total counts incorporated from uridine appears in the RNA fraction, while the products of thymidine feeding appears not only in DNA but about 46% in protein. About 90% of leucine incorporated into protein. Many attempts were made to alleviate cross contamination to increase the recovery of the label in the desired fraction. We could not improve the condition by various combinations of changing the treatment periods in acid either at cold or at elevated temperatures, nor by washing the respective pellets carefully several times. These initial situation prompted us to develop an alternative method.

During the development of the first enzymatic method, the use of a protease, Nagase, was attempted. However, the protease was found not to be able to solubilize all perchloric acid precipitable proteins under several different experimental conditions. In addition, protein precipitate is much larger than nucleic acid precipitates. In view of these results, the use of protease was discontinued. Although radioactivity from a precursor can be found in all three fractions even with the use of DNase and RNase, the cross contamination can be much reduced by employing proper amount of enzymes and incubation time. With the first enzymatic method, the incorporation of ^{3}H-thymidine into the RNA fraction can be reduced much less than 20%, and that of ^{14}C-uridine into the DNA

Table VI-2. Incorporation of precursors into RNA, DNA, and protein fractions. 10 root tips were treated with Clorox, washed thoroughly, and incubated with ^{14}C-leucine (10^6 CPM), ^{14}C-uridine (5×10^5 CPM), or ^3H-thymidine (10^6 CPM) for 2 hours. RNA, DNA, and protein fractions were made by the perchloric acid procedure of Woodstock and Skoog (1960)

Precursor administered	Radioactivity in fraction of					
	RNA		DNA		Protein	
	CPM	%*	CPM	%*	CPM	%*
^3H-thymidine	0	0	13,000	54	10,950	46
^{14}C-uridine	8,920	43	7,050	34	4,930	23
^{14}C-leucine	9,300	3	21,120	7	288,500	90

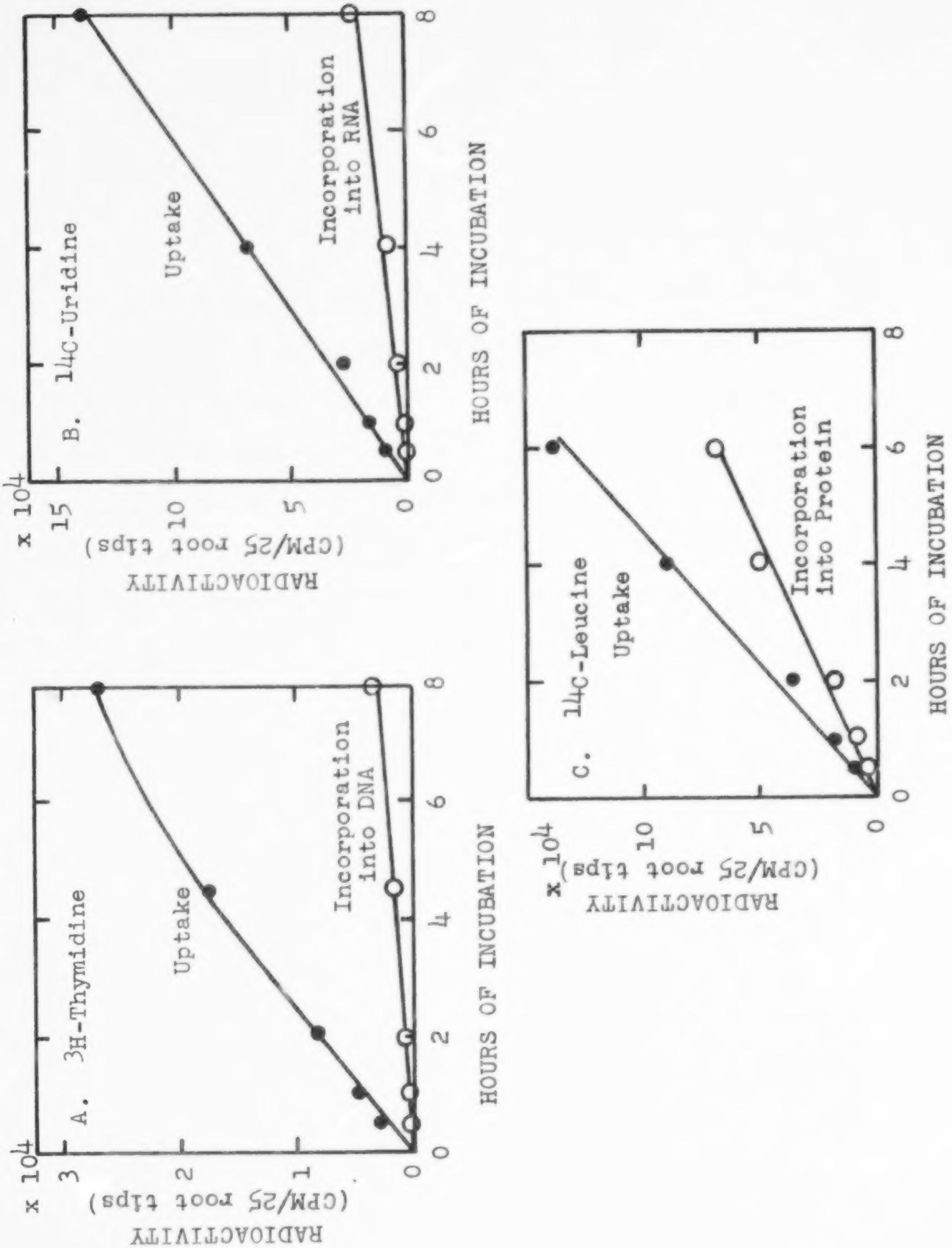
* % of total counts incorporated.

fraction to about 20%. Some radioactivity appeared in the protein fraction from the nucleic acid precursors, but ^{3}H-thymidine incorporation into protein can be easily substracted by the use of the tritium window from the count with the window for both tritium and ^{14}C. This situation allowed us to carry out a double labeling procedure using ^{3}H-thymidine and ^{14}C-leucine, separate from ^{14}C-uridine, but simultaneous labeling of all three fractions with respective precursors was concluded to be unrealistic. It should be added that the cross contamination is not simply due to adhesion of the radioactive precursors to fractions, because an addition of radioactive precursors to homogenized root tips did not make any fraction radioactive.

The first enzymatic method was examined in various ways. Time course incorporation and uptake of radioactive precursors, when singly applied, are shown in Fig. VI-1. The uptake was the sum of radioactivity found in all steps of extraction. Both incorporation and uptake are linear during the first several hours. The uptake proceeds about 0.6% of the administered radioactivity per hour for thymidine, about 1.6% per hour for uridine, and about 2.2 % per hour for leucine. The per cent incorporation of radioactivity taken up is about 8% for thymidine, about 14% for uridine, and about 50% for leucine. In terms of the initial radioactivity administered the amount of radioactivity detectable after 2 hours of incubation is far less than 1% for nucleic acid precursors and about 2% for leucine. The low levels of incorporation pose a question as to whether the radioactivity measurement

Fig. VI-1. Time course of uptake and incorporation of precursors into respective polymers.

The uptake is the sum of radioactivity found in all steps of extraction. The incorporation was the counts in the expected polymer fraction. Initial radioactivity administered: 7 x 10^5 CPM for thymidine; 1 x 10^6 CPM for uridine and leucine. The first enzymatic method was used in these experiments.


A. ^{3}H-Thymidine
Uptake
Incorporation into DNA
RADIOACTIVITY (CPM/25 root tips)
x 10^4
0
1
2
3
HOURS OF INCUBATION
0
2
4
6
8
B. ^{14}C-Uridine
Uptake
Incorporation into RNA
RADIOACTIVITY (CPM/25 root tips)
x 10^4
0
5
10
15
HOURS OF INCUBATION
0
2
4
6
8
C. ^{14}C-Leucine
Uptake
Incorporation into Protein
RADIOACTIVITY (CPM/25 root tips)
x 10^4
0
5
10
HOURS OF INCUBATION
0
2
4
6
8

is indeed valid to assess the activities of protein and nucleic acid syntheses.

In order to examine the above question, we investigated the possibility of competition for incorporation by non-radioactive precursors. When root tips are incubated with a fixed concentration of a radioactive precursor together with varied amounts of the same precursor without radioactivity, the rate of incorporation of radioactivity into the expected polymer should decrease after the total concentration of the precursor exceeds a certain value. Our experiment showed the expected results (Fig. VI-2). This indicates that the radioisotope and non-radioisotope of the same precursor are incorporated equally into the polymer and that, as the fraction of radioactivity in the total concentration decreases, the incorporation of radioisotope becomes less and that of non-radioisotope increases. Although not shown here, the uptake of radioactivity by root tips showed the same pattern as shown in Fig. VI-2. Thus, non-radioactive precursors are capable of competing with the radioactive precursors for both incorporation and uptake.

The results from the above competition experiment and also hydrolysis by purified DNase and RNase of respective nucleic acids strongly indicate that the enzymatic method detects the incorporation of radioactive precursors into respective polymers (i.e., the activities of root tip cells to synthesize protein and nucleic acids).

In order to increase the efficiency of incorporation

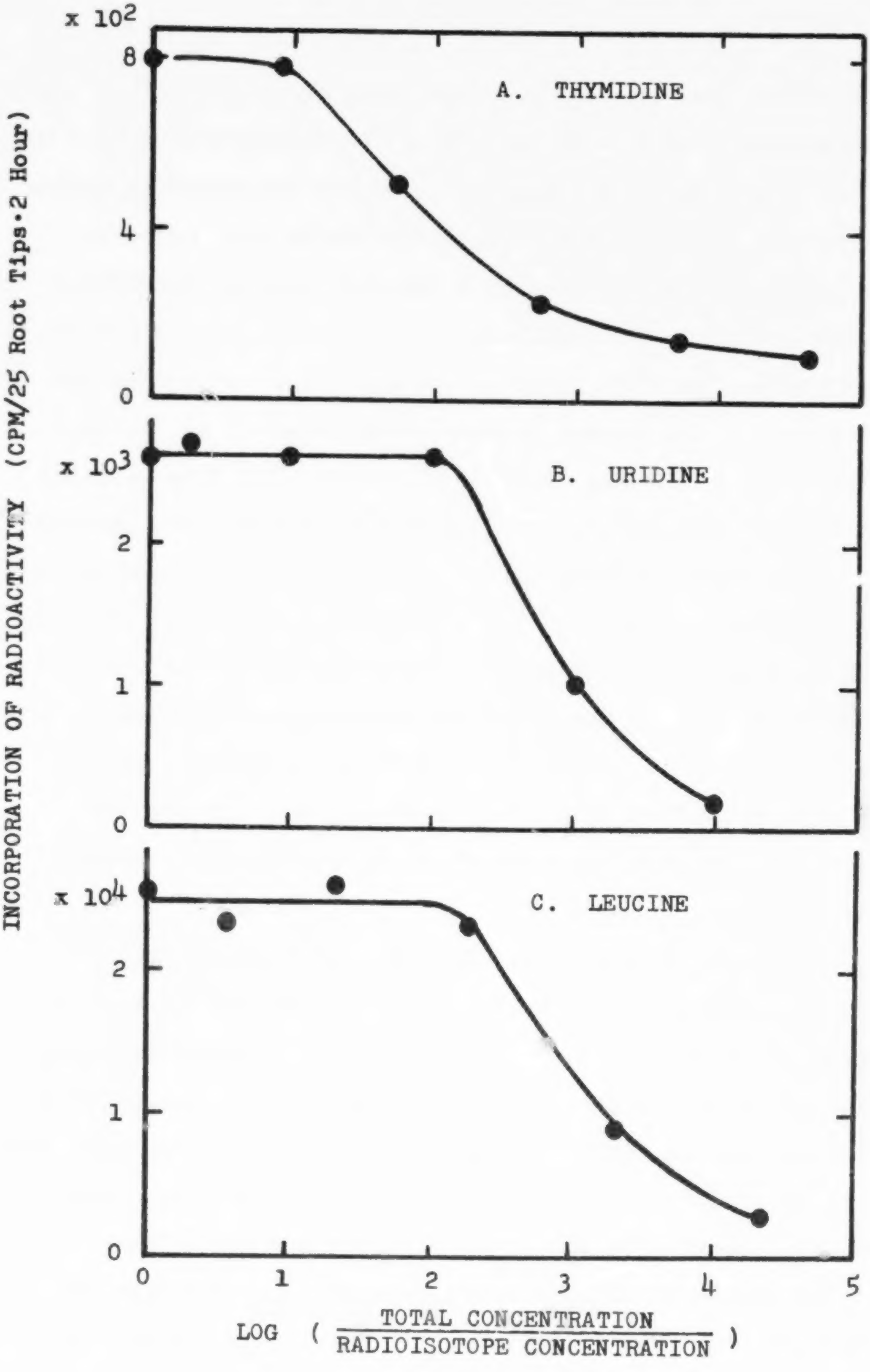
Fig. VI-2. Competition of non-radioactive precursors with radioactive ones for incorporation into respective polymers in corn root tips.

A. Competition of non-radioactive thymidine with ^{3}H-thymidine for incorporation into DNA.

B. Competition of non-radioactive uridine with ^{14}C-uridine for incorporation into RNA.

C. Competition of non-radioactive leucine with ^{14}C-leucine for incorporation into protein.

In these experiments, 25 root tips were incubated for 2 hours in 5 ml medium containing a precursor and 50 mM glucose, and the pellet from the homogenization step was used for radioactivity measurements. The concentration of radioactive precursor was kept constant for each series: thymidine, 40 nM (7 x 10^5 CPM); uridine, 200 nM (10^6 CPM); leucine, 100 nM (10^6 CPM). Non-radioactive precursors were added over ten thousand fold excess of the radioactive precursors; in order to cover the wide range of concentrations, the abscissa is expressed in Log ratio of total to radioisotope concentration.


x 10²
8
4
0
A. THYMIDINE
INCORPORATION OF RADIOACTIVITY (CPM/25 Root Tips·2 Hour)
x 10³
2
1
0
B. URIDINE
x 10⁴
2
1
0
C. LEUCINE
0
1
2
3
4
5
LOG (TOTAL CONCENTRATION / RADIOISOTOPE CONCENTRATION)

and reduce time consumption, the first enzymatic method was re-examined. With corn root tips, the de-lipidation step was found unnecessary, and incorporation was increased by either increasing the radioactivity in the incubation medium or keeping the same radioactivity but reducing the incubation volume. These considerations led us to the development of the second, improved enzymatic method which emphasizes the reduction of incubation volume and elimination of the de-lipidation step. Figure VI-3 illustrates the time course of uptake and incorporation of thymidine, uridine, and leucine with the improved method. Uptake, incorporation, and per cent incorporation of radioactivity taken up by the root tips are all increased by this method. With respect to the administered radioactivity, the amount of incorporation of precursors into respective polymers were about 0.8% per hour for thymidine incorporation into DNA, about 1.2% per hour for uridine incorporation into RNA, and about 6% per hour for leucine incorporation into protein.

In order to anticipate possible inhibition of protein and/or nucleic acid syntheses by herbicide, the effects of known inhibitors of these processes were examined with the improved method (Table VI-3). Hydroxyurea, an inhibitor of DNA replication, inhibits the incorporation of thymidine into DNA; the incorporation of other precursors and the uptake of all precursors were not affected. Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, specifically inhibits the incorporation of uridine into RNA, but does not affect the

Fig. VI-3. Time course of uptake and incorporation of precursors into respective polymers in corn root tips. Uptake is the sum of radioactivity found in all fraction of extraction. Incorporation is the counts in expected polymer fractions; i.e., thymidine into DNA, uridine into RNA, and leucine into protein. Initial radioactivity administered: 7×10^5 CPM for thymidine, 10^6 CPM for uridine and leucine. The second (improved) enzymatic method was used for extraction. % incorporation indicates the amount of radioactivity incorporated from the amount taken up by the root.

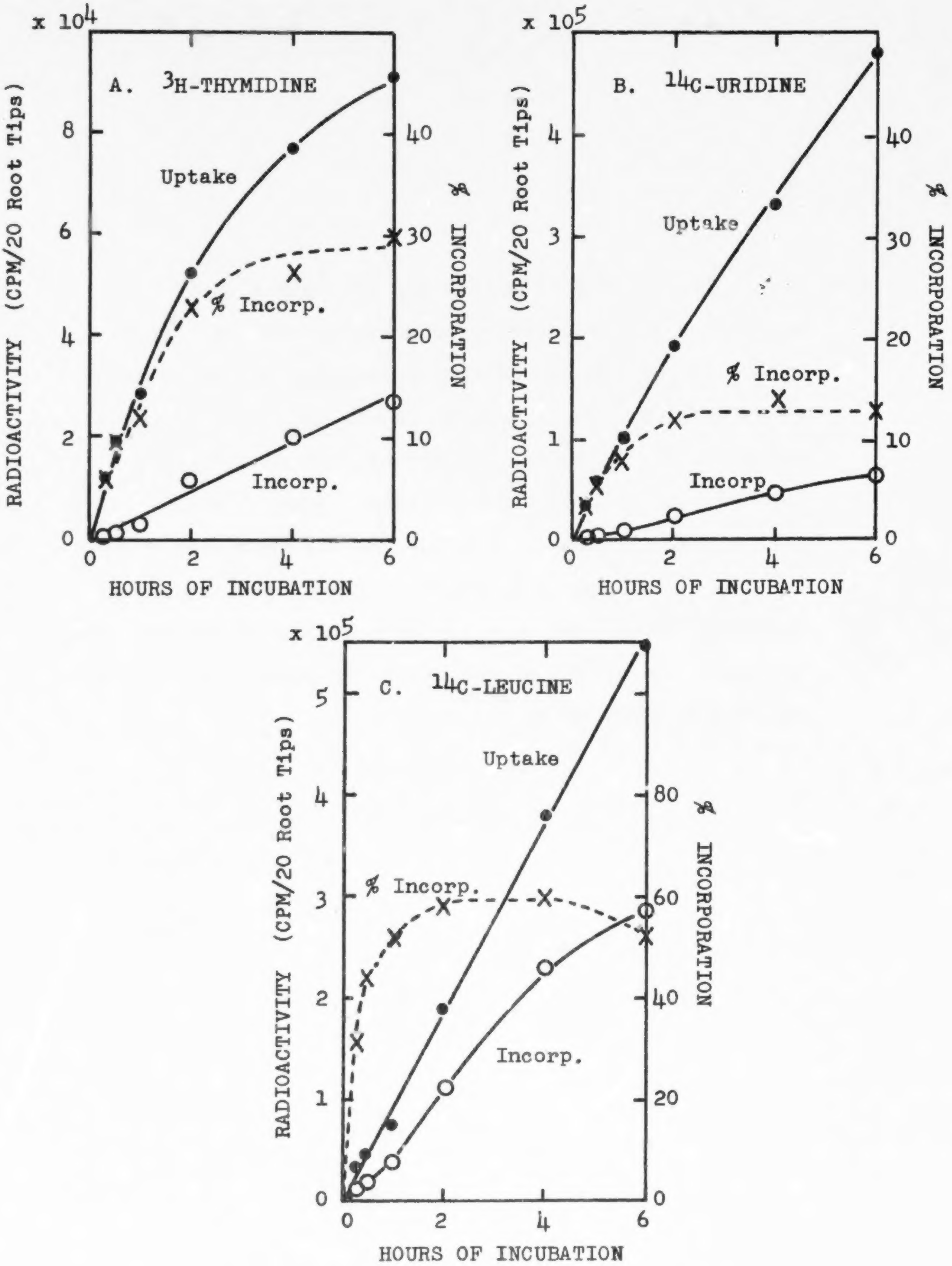

x 10^4
A. 3H-THYMIDINE
Uptake
% Incorp.
Incorp.
RADIOACTIVITY (CPM/20 Root Tips)
% INCORPORATION
0
2
4
6
8
0
10
20
30
40
HOURS OF INCUBATION
0
2
4
6
x 10^5
B. 14C-URIDINE
Uptake
% Incorp.
Incorp.
RADIOACTIVITY (CPM/20 Root Tips)
% INCORPORATION
0
1
2
3
4
0
10
20
30
40
HOURS OF INCUBATION
0
2
4
6
x 10^5
C. 14C-LEUCINE
Uptake
% Incorp.
Incorp.
RADIOACTIVITY (CPM/20 Root Tips)
% INCORPORATION
0
1
2
3
4
5
0
20
40
60
80
HOURS OF INCUBATION
0
2
4
6

Table VI-3. Effects of known inhibitors of protein and nucleic acid syntheses on incorporation and uptake. The second enzymatic method was employed with 20 root tips per treatment.

Inhibitor*	INCORPORATION into						UPTAKE by root tips					
	D N A		R N A		Protein		Thymidine		Uridine		Leucine	
	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control
Hydroxyurea												
0	11,000	100	17,600	100	77,900	100	45,000	100	175,000	100	136,000	100
10^{-4}	11,000	100	17,600	100	79,000	101	42,800	95	170,000	97	130,000	96
10^{-3}	8,050	73	16,200	92	88,300	114	47,100	105	163,000	93	145,000	107
$5x10^{-3}$	4,170	38	16,800	95	91,600	118	47,000	105	164,000	94	141,000	104
Actinomycin D												
0	7,700	100	5,100	100	53,100	100	33,600	100	140,000	100	97,200	100
$8x10^{-5}$	10,100	131	2,015	40	67,500	127	40,200	120	125,000	89	118,000	122
$3x10^{-4}$	8,300	108	1,100	22	68,300	128	37,100	110	120,000	86	116,000	119
Cycloheximide												
0	20,200	100	33,700	100	121,000	100	70,200	100	275,000	100	226,000	100
10^{-5}	10,700	53	32,700	97	6,480	5	47,700	68	262,000	95	164,000	73
10^{-4}	11,100	55	33,500	99	4,380	4	47,100	67	256,000	93	177,000	78
10^{-3}	9,600	47	30,500	94	3,530	3	44,600	64	228,000	83	155,000	69

* Numbers in this column denote molar concentrations.

incorporation of other precursors. The uptake of uridine into root tips is slightly inhibited by actinomycin D, while the uptake of other precursors is somewhat stimulated. Cycloheximide, an inhibitor of protein synthesis at the translational step, strongly inhibits protein synthesis at 10^{-5} M. It exerts some inhibition of uptake of all precursors; this inhibition is reflected in slight inhibition of incorporation of thymidine into DNA. These expected results further support the validity of enzymatic methods in examination of protein and nucleic acid syntheses.

It should be noted in Table VI-3 that the amounts of radioactivity taken up by root tips and those incorporated into respective polymers differ from experiment to experiment widely. In a number of attempts, this variation was found very difficult to overcome. However, experimental variations within the same experiment using the same batch of root tips were small usually within 10 to 20%. These observations strongly suggest that the physiological conditions of root tips are relatively uniform within each batch, but the conditions of root tips from one batch to another are not identical, although each batch of corn seedlings was grown practically in an identical manner with the rest. Therefore, we adopted a procedure to use the same batch of roots for a given series of experimental runs and the comparison of treatment be made on the basis of relative effect; i.e., on the basis of per cent of control (or no treatment).

Herbicidal Effects on Protein and Nucleic Acid Synthesis. Phytotoxicity of some herbicides was at first examined with the first enzymatic method using 25 root tips. With this method, the emphasis was placed on the examination of the effects of herbicides on the incorporation of radioactive precursors into DNA, RNA, and protein. With the development of the second, more reliable method, all but three herbicides were screened by this modified method. The number of root tips used for each run was set to 20 and the incubation time was 2 hours with radioisotopes. Herbicide was given 30 minutes before introduction of herbicide. Uridine was administered singly, while leucine and thymidine were given together. In addition, herbicidal effects were examined not only on the incorporation of radioactive precursors to DNA, RNA, and protein fractions, but also on the uptake of radioactive precursors into root tips. The herbicide concentrations used were 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M. With most herbicides, the limit of solubility with 1% or less DSMO as the solvent was 10^{-4} M. Since 10% DMSO is inhibitory for both uptake and incorporation, the data with 10% DMSO will not be presented. The results of the survery of phytotoxic effects of 17 herbicides are given in Table VI-4, where each reading is an average of duplicate runs. It should be mentioned that the concentrations of herbicides in a lower range which did not show any detectable effects are not included in this table.

Of the seventeen herbicides tested, five herbicides did not affect either the uptake or the incorporation appreci-

Table VI-4. Effects of 17 herbicides on protein and nucleic acid syntheses.

Both incorporation of radioactive precursors into DNA, RNA, and protein fractions and uptake of radioactive thymidine, uridine, and leucine are given for each herbicide treatment.

Herbicide*	INCORPORATION into						UPTAKE by root tips					
	D N A		R N A		Protein		Thymidine		Uridine		Leucine	
	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control
Amitrole												
0	20,500	100	39,900	100	118,000	100	60,500	100	312,000	100	221,000	100
10^{-3}	24,300	119	48,600	122	151,000	128	61,300	101	329,000	106	271,000	123
Atrazine												
0	12,900	100	22,400	100	175,000	100	46,400	100	232,000	100	279,000	100
10^{-4}	13,000	101	17,100	76	163,000	93	42,500	92	177,000	76	249,000	90
Bromacil												
0	10,200	100	19,900	100	103,000	100	40,700	100	178,000	100	160,000	100
10^{-3}	2,700	26	3,600	18	92,000	89	22,700	56	32,800	18	139,000	87
Cacodylate												
0	16,200	100	36,300	100	92,000	100	67,000	100	299,000	100	175,000	100
10^{-3}	17,800	110	38,300	105	113,000	123	67,000	100	270,000	90	172,000	98
Chlorpropham												
0	13,400	100	17,400	100	83,200	100	42,100	100	190,000	100	144,000	100
4×10^{-4}	9,300	70	11,800	68	57,000	69	34,300	82	129,000	68	105,000	73

Table VI-4 --- continued

Herbicide*	INCORPORATION into						UPTAKE by root tips					
	D N A		R N A		Protein		Thymidine		Uridine		Leucine	
	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control
Diallate												
0	10,200	100	19,900	100	103,000	100	40,700	100	178,000	100	160,000	100
10^{-4}	9,800	96	18,900	95	102,000	99	39,300	97	163,000	92	154,000	96
Diquat												
0	13,200	100	44,700	100	112,000	100	64,000	100	380,000	100	195,000	100
10^{-5}	18,300	139	44,700	100	113,000	101	66,000	103	378,000	100	207,000	106
10^{-4}	17,800	135	28,300	63	87,000	78	60,000	94	301,000	79	170,000	87
10^{-3}	8,400	64	20,700	46	37,000	33	49,000	77	231,000	61	114,000	58
Diuron												
0	8,700	100	28,700	100	81,700	100	32,400	100	170,000	100	115,000	100
10^{-4}	8,900	102	22,900	80	93,700	114	32,400	100	134,000	79	129,000	112
DNOC**												
0	12,900	100	1,580	100	104,000	100						
$2x10^{-5}$	5,180	40	1,080	68	18,700	18						
$2x10^{-4}$	60	0.5	320	20	200	0.2						
0	24,500	100	33,900	100	91,900	100	70,700	100	231,000	100	193,000	100
10^{-4}	4,390	18	3,330	10	6,520	7	21,700	31	56,800	25	40,200	21
DSMA												
0	18,300	100	37,500	100	153,000	100	79,300	100	293,000	100	260,000	100
10^{-3}	25,000	136	32,200	99	173,000	113	79,000	100	253,000	86	279,000	107

Table VI-4 --- continued

Herbicide*	INCORPORATION into						UPTAKE by root tips					
	D N A		R N A		Protein		Thymidine		Uridine		Leucine	
	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control
Karbutylate**												
0	4,300	100	1,970	100	21,500	100						
10^{-4}	4,660	108	1,820	92	28,500	133						
10^{-3}	3,260	76	1,300	66	24,200	113						
Monuron**												
0	4,630	100	2,450	100	25,200	100						
10^{-4}	5,210	113	1,150	47	30,000	119						
10^{-3}	2,780	60	650	27	27,200	108						
Propham												
0	16,500	100	37,800	100	110,000	100	68,500	100	274,000	100	182,000	100
10^{-4}	15,100	91	34,200	90	126,000	115	59,000	86	230,000	84	210,000	115
10^{-3}	7,760	47	14,400	38	44,000	40	39,700	58	90,400	33	83,700	46
Simazine**												
0	3,280	100	2,040	100	16,000	100						
10^{-4}	3,080	94	1,700	83	17,500	110						
Trifluralin												
0	10,200	100	19,900	100	103,000	100	40,700	100	178,000	100	160,000	100
10^{-4}	10,700	105	23,800	120	116,000	113	40,300	99	206,000	116	170,000	106

Table VI-4 --- continued

Herbicide*	INCORPORATION into						UPTAKE by root tips					
	D N A		R N A		Protein		Thymidine		Uridine		Leucine	
	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control	CPM	% of control
2,4-D												
0	19,300	100	42,000	100	142,000	100	60,800	100	346,000	100	228,000	100
10^{-4}	19,700	102	42,000	100	123,000	87	54,700	90	323,000	93	181,000	79
10^{-3}	3,600	19	4,600	11	8,500	6	22,700	37	118,000	34	32,000	14
2,4,5-T												
0	10,200	100	29,700	100	83,800	100	58,200	100	225,000	100	175,000	100
10^{-4}	14,100	138	27,500	93	120,000	143	45,100	78	208,000	93	191,000	109
$5x10^{-4}$	5,300	52	18,000	61	6,500	8	16,200	28	138,000	61	30,800	18

* Numbers in this column denote molar concentrations of herbicide used.

**Experiments without uptake data were done by the use of the first enzymatic method with 25 root tips. All other expements were done with the second enzymatic method using 20 root tips.

ably. They are amitrole, cacodylate, diallate, DSMA, and trifluralin. In some cases, slight stimulation of uptake and/or incorporation was noted. At the limit of solubility, atrazine, diuron, and simazine caused slight inhibition of RNA synthesis. Karbutylate and monuron inhibited syntheses of both DNA and RNA significantly at 10^{-3} M; inhibition of RNA synthesis is stronger than that of DNA synthesis. The remaining seven herbicides (bromacil, chlorpropham, diquat, DNOC, propham, 2,4-D, and 2,4,5-T) affected the syntheses of DNA, RNA, and protein to various degrees. Amongst these, DNOC was the most potent inhibitor of these synthetic processes, affecting incorporation at 10^{-5} to 10^{-4} M. 2,4-D exerted strong inhibition of all three processes only at 10^{-3} M. Only at the highest concentrations used, chlorpropham and propham inhibited all three processes equally by about 50%, while bromacil inhibited nucleic acid synthesis more strongly than protein synthesis, and diquat and 2,4,5-T inhibited protein synthesis more strongly than nucleic acid synthesis.

If one compares the incorporation and uptake data, one notes a marked correlation between the inhibition of incorporation and that of uptake. Herbicides which cause inhibition of one or more synthetic processes also cause inhibition of uptake of the corresponding radioactive precursors. Since the precursors taken up by the root tip cells are incorporated into respective polymers, these herbicides can be considered to affect primarily the uptake of precursors. The effects of these herbicides are thus different from those of known inhibitors of protein and nucleic acid syntheses (Table VI-3).

DISCUSSION

During the course of this investigation, three methods were examined. The second enzymatic method is far superior to the other two. This method takes less time and causes less variations than the other two methods. With the incubation period of two hours, corn root tips incorporate high radioactivity into DNA, RNA, or protein (Figs. VI-1 and VI-3). The competition experiment (Fig. VI-2) and the study with known inhibitors (Table VI-3) indicate that the enzymatic method detects expected syntheses of protein and nucleic acids. In order to improve the method further, we made repeated attempts to label DNA, RNA, and protein with simultaneous application of radioactive thymidine, uridine, and leucine. These attempts resulted in failure, mainly because of incorporation of nucleic acid precursors into protein fraction and protein precursor into nucleic acid fractions. We thus decided to label RNA singly with uridine and to double-label DNA and protein with thymidine and leucine, respectively. Distribution of radioactivity in all fractions in single applications is presumably due to the metabolism of the precursor by the root tip cells during incubation. In view of this problem, enzymatic hydrolysis of nucleic acids is important in analysis of all three synthetic processes. The enzymes to be employed must be specific to either DNA or RNA, devoid of proteolytic activity.

Biological variations from experiment to experiment were large in our analyses (Tables VI-3 and VI-4). In our microscopic observations, about 5 to 15% of root tip cells are undergoing mitosis. It is highly likely that the mitotic and other metabolic activities in the root tip cells follow some endogenous rhythms. Inspite of our repeated attempts, we could not regulate the biological variations. With the same batch of seedlings, however, experimental errors were reduced to a tolerable range of 10 to 20%.

The survey of herbicidal effects on the uptake of radioactive thymidine, uridine, and leucine into the root tip and on the incorporation of these precursors into DNA, RNA, and protein, respectively, show a clear correlation between uptake and incorporation (Table VI-4). These observations are clearly different from the action of known inhibitors of protein and nucleic acid syntheses (Table VI-3). With the herbicides employed, it was thus concluded that their primary effect must be on the uptake process rather than on the syntheses of protein and nucleic acids. In addition, with a lone exception of DNOC, inhibitory effects were seen at the highest concentrations corresponding to the limit of solubility of effective herbicides. This point further lead to a possibility that their effects on uptake may not be the primary action of these herbicides (see other Chapters). In the case of DNOC, it acts as a potent uncoupler of oxidative phosphorylation of isolated mitochondria at 20-30 μM (Chapter II), as a potent inhibitor of electron transport in isolated chloroplasts (Chapter III), and as a stimulator of respiration in isolated corn root tips (Chapter IV). The effective concentration of this herbicide on the uptake of radioactive precursors and their incorporation into nucleic acids and protein is in the same range as for the above mentioned processes. It is highly likely that this herbicide penetrates into the root cells and acts on mitochondria to uncouple oxidative phosphorylation, thus depleting the endogenous ATP level. The depletion of ATP probably reduces the uptake of radioactive thymidine, uridine, and leucine (cf. Ashton and Crafts, 1973).

These interpretations of the results presented in Table VI-4 clearly point out that the analyses of protein and nucleic acid syntheses by means of incorporation of radioactive precursors must be accompanied by the analysis of uptake of the precursors by the tissue under study. The incorporation study is an indirect way to examine syntheses and depends on the amounts of precursors available in the cell. Protein and nucleic acid syntheses are not simple processes: they depend, like any cellular processes, on many other cellular activities. It is highly probable that an inhibitor of other cellular activities inhibits protein and nucleic acid metabolism, when intact tissues are employed. In order to assess possible phytotoxic acition of herbicides in protein and nucleic acid syntheses using intact tissues, information on the action of known inhibitors of these synthetic processes is indeed valuable (Table VI-3). From these considerations, we conclude that all 17 herbicides tested are not the primary inhibitors of protein and nucleic acid syntheses.

EVALUATION OF METHOD

We have developed an enzymatic method to analyze possible phytotoxic action of herbicides on protein and nucleic acid syntheses using corn root tips. The method is a indirect way to examine herbicidal action. In order to use this method effectively, we recommend to obtain information not only about the incorporation of radioactive precursors into DNA, RNA, and protein fractions, but also about the uptake of the precursors by the tissue. The data must also be compared with the action of specific inhibitors of these synthetic processes. Since a given herbicide may cause multiple effects on cellular processes, the availability of information about how it acts on other cellular processes will no doubt aid the final assessment of whether the herbicide acts directly and specifically or indirectly on protein and nucleic acid syntheses.

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CHAPTER VII

EFFECTS OF HERBICIDES ON ULTRASTRUCTURE OF CORN ROOT TIP CELLS AND SOYBEAN LEAF CELLS

By Robert J. Lowry and Frank Begun

INTRODUCTION

Important vital functions of the plant cell are carried out on cellular and subcellular membranes, such as transport, compartmentation, electron transport and associated phosphorylation, and enzymatic syntheses of cell wall components (Anderson and Thomson, 1973). Several inhibitors of mitochondrial function exert their inhibitory effects by irreversibly and stoichiometrically binding with mitochondrial membranes (cf. Ikuma and Bonner, 1967a,b; Lehninger, 1965). Similarly, diallate has been demonstrated in this report to bind with mung bean hypocotyl mitochondria and corn root mitochondria irreversibly and stoichiometrically (CHAPTER II). Some, if not many, herbicides are very poorly soluble in water and are not readily transported in the plant, and their effects are often local (cf. Ashton and Crafts, 1973). In addition, many herbicides are inhibitors of photosynthetic electron flow, particularly at photosystem II (CHAPTER II; Ashton and Crafts, 1973; Moreland, 1967). These observations raise a possibility that some herbicides act to alter cellular and subcellular membranes structurally.

Herbicides can be considered to cause accelerated senescence which is accompanied by alteration and disruption of cellular organization (Anderson and Thomson, 1973). The phenoxy type herbicides are known to cause massive damage in cellular ultrastructure (Hallam, 1970). The bipyridilium type of herbicides alters the integrity of cellular membrane (Harris and Dodge, 1972). Trifluralin acts to interrupt mitosis by disrupting spindle microtubules (Bartels and Hilton, 1973). Spindle microtubules are shown to be disoriented by propham (Hepler and Jackson, 1969). Apparently herbicidal treatments do not affect much the ultrastructure of nucleus, mitochondria, and microbodies, but strongly influence in a short period of treatment the ultrastructure of plastids, mitosis, and cell wall formation (Anderson and Thomson, 1973).

In our tissue respiration and photosynthesis study (CHAPTERS IV and V), we have observed relatively fast responses of isolated tissues, particularly bean leaf disks, to herbicide treatments. It is, therefore, of interest to examine whether the observed physiological responses are accompanied by changes in the cellular ultrastructure of the tissues. In the present study we employed corn root tips and mature soybean leaf tissue. This study enables us not only to provide ultrastructural bases to the biochemical and physiological studies thus far reported, but also to compare herbicidal effects on differentiating cells with those on mature, well differentiated cells.

MATERIALS AND METHODS

Preparation of Plant Materials. Plant materials were prepared in a matter comparable to earlier studies.

Corn root tips (3 mm in length) were excised from the primary roots of Michigan Hybrid 200 corn with a cutting device, placed in a herbicidal solution, and aerated for 2 hours in a practically identical manner to the studies with root tip respiration and protein synthesis. Immediately after 2 hours of incubation in aerated herbicide solution, 1 mm root tips were prepared and fixed.

From the first or second fully developed trifoliate soybean leaf, shredded leaf sections (about 1 mm in width) were prepared, vacuum-infiltrated with a treatment solution, and incubated in light for 2 hours. Small pieces were cut from these sections and fixed.

In both root tip and leaf studies, 6 to 10 pieces were fixed for each concentration. Concentrations of herbicides used were in the range of 10^{-3} to 10^{-6} M.

Preparation of Thin Sections. The following steps were employed in preparation of thin sections:

a. Fixation of plant material with 4% glutaraldehyde for 2 hours at room temperature. The fixative was removed by washing with 3 to 5 changes of 0.05 M cacodylate buffer at pH 7.3 in one hour.

b. Post-fixation with 2% osmium tetraoxide in 0.05 M cacodylate buffer at 4° C for 1½ hours. The plant material was rinsed with the cacodylate buffer for one hour with 3 changes of the rinsing buffer.

c. Dehydration of the fixed material in an ethanol series (15%, 30%, 50%, 70%, 95%, and 3 times in 100%) for 15 minutes in each step.

d. Treatment with propylene oxide 2 times for 15 minutes each, prior to embedding.

e. Infiltration with the embedding resin mixture (epon resin mixture : propylene oxide = 1 : 2) overnight in a dessicator. Plant materials were then transferred to gelatin capsules containing fresh embedding resin mixture and placed in a 78° oven overnight.

f. Sectioning of the plant materials with a LKB ultramicrotome using a glass knife to about 60 nm thickness.

g. Staining for 30 minutes with 0.5% uranylacetate, followed by Reynold's lead citrate for 2 minutes.

The prepared sections were examined by a RCA or a Zeiss electron microscope. Microscopic observation was routinely done from the highest concentration (10^{-3} M) to the concentration at which no significant effect was observable.

RESULTS

Herbicide Effects on the Ultrastructure of Corn Root Tip Cells. Prior to examining herbicidal effects, the morphology of control cells was investigated. Three controls examined were root tip cells after two hours of water treatment, after 2 hours of 1% DMSO treatment, and 10% DMSO treatment. One per cent DMSO was employed for 10^{-4} M solution of poorly water insoluble herbicides and 10% for 10^{-3} M solutions of the same herbicides. Electron photomicrographs for water control show normal cell plate formation and associated spindle microtubules (Plate 1), normal mitochondria with cristae (Plates 1 and 2), normal plastids (Plates 1 and 2), normal nuclei (Plate 1), normal vacuole (Plate 2), and normal cell wall with plasmodesma and associated wall microtubules (Plate 2). One per cent DMSO control showed essentially the same pictures as those for water control. Treatment with 10% DMSO frequently resulted in separation of adjacent cells at the middle lamella (not shown) and somewhat inflated mitochondria (Plate 3), but ultrastructures of other organelles and membranes are practically identical with water control cells. In scanning many control cells, we observed very rarely one or two, out of hundreds, enlarged, donuts- or ring-shaped mitochondria. After establishment of the ultrastructure of control cells, alterations of morphology in herbicide-treated cells were examined. Although observations were made on all observable organelles, abnormalities were detected in mitotic figures, cell plate formation, mitochondrial and plastid structure, cell wall and associated microtubules, vacuolation of cytoplasm. We have not detected particular abnormalities in the ultrastructure of nuclei, golgi apparatus, and ribosomes. A summary of herbicidal effects is given in Table VII-1.

Table VII-1. Summary of effects of 17 herbicides on the ultrastructure of corn root tip cells.

..... denotes normal appearance at all concentrations tested. Concentrations at which effects are observed are given in parentheses.

HERBICIDE	Mitochondria	Plastids	Cell Wall	Mitotic Figure	Other Effects
Amitrole	.....	.....	Somewhat beady appearance (10^{-3}M)	.....	.....
Atrazine	Enlarged, donuts-shaped (10^{-5}M)	.....	.....	.....	Excessive vacuolation (10^{-3}M)
Bromacil	Enlarged, donuts-shaped (10^{-3}-10^{-6}M)	.....	.....	.....	.....
Cacodylate	Enlarged, some donuts (10^{-3},10^{-4}M)	.....	.....	.....	.....
Chlorpropham	.....	.....	Fewer microtubules (10^{-4}M)	Abnormal spindle orientation & cell plate formation (10^{-5},10^{-4}M)	.....
Diallate	.....	.....	Somewhat beady (10^{-3}M)	No mitotic figure observed (10^{-3}M)	.....
Diquat	.....	.....	.....	.....	Excessive vacuolation (10^{-4}, 10^{-5}M)
Diuron	Enlarged, barbell shaped (10^{-4}M)	Slightly elongated (10^{-4}M)	.....	.....	.....
DNOC	.....	.....	.....	Occasionally cell plate missing (10^{-4}, 10^{-5}M)	Excessive vacuolation (10^{-4}, 10^{-5}M)

Table VII-1 --- continued

HERBICIDE	Mitochondria	Plastids	Cell Wall	Mitotic Figure	Other Effects
DSMA	Occasional donuts, slightly enlarged cristae (10^{-3},10^{-4}M)	Ring-shaped with enlarged starch & lamellae (10^{-3}M)	.....	.....	More osmophilic bodies in cytoplasm (10^{-3}M)
Karbutylate	.....	.....	.....	Irregular & incomplete cell plate formation. Multinucleate cells. (10^{-3}M)	.....
Monuron	Enlarged, donuts shaped (10^{-4},10^{-5}M)	......	.....	.....	.....
Propham	Enlarged, donuts shaped (10^{-4}, 10^{-5}M)	Expanded lamellae (10^{-4}M)	.....	Abnormal spindle orientation & cell plate formation (10^{-4}, 10^{-5}M)	.....
Simazine	.....	.....	.....	Irregular cell plate formation (10^{-4}M)	.....
Trifluralin	.....	.....	Fewer microtubules (10^{-5}M)	No formation of spindle and cell plate (10^{-5}M)	.....
2,4-D	Donuts-shaped (10^{-3}M)	Ring-shaped (10^{-3}M)	.....	No telophase figures, no cell plate (10^{-3}M)	Massive cell damage (10^{-3}M)
2,4,5-T	.....	Ring-shaped, slight elongation (10^{-4}M)	.....	.....	Massive cell damage (10^{-3}M)

Amitrole is a relatively ineffective herbicide. Only at 10^{-3} M only possible effect observed was on the appearance of of the cell wall; the wall showed increased indentations even to a beady appearance (Plate 4). Other structures were practically identical to the control pictures.

Eight herbicides affected the morphology of mitochondria strikingly; mostly causing ring-shape in appearance. They are atrazine (relatively weak effect; photomicrograph, not shown), bromacil (Plate 6), cacodylate (elongated mitochondria shown in Plate 7), diuron (elongated mitochondria shown in Plate 13), DSMA (swollen mitochondria shown in Plate 17), monuron (swollen and elongated mitochondria shown in Plate 20), and propham (ring-shaped mitochondria in plate 22 and excessively elongated mitochondria in plate 23). Although not shown in photomicrographs, 2,4-D treatment resulted in ring-shaped mitochondria. Bromacil and cacodylate affected only the mitochondrial structure and no effect was observable on other organelles. In terms of concentrations, bromacil was the most potent herbicide in alteration of mitochondrial morphology.

Three, possibly five, herbicides affected the ultrastructure of plastids. They are diuron (elongated plastids in Plate 13), DSMA (elongated, ring-shaped plastids in Plate 16), and propham (abnormal, ring-shaped plastids in Plate 21). 2,4-D and 2,4,5-T caused altered plastids (Plate 25 for 10^{-4} M 2,4,5-T treatment), but these herbicides appear to induce excessive cell damage (Plates 9,10, 26) at 10^{-3} M and their effects on the structures of other organelles may be interpreted as the effects prior to massive cell damage.

Alteration of cell division is caused by 8 herbicides. Amongst them, diallate at 10^{-3} M (Plate 11), DNOC at 10^{-4} and 10^{-5} M (Plate 15), trifluralin at 10^{-5} M (Plate 27), and 2,4-D at 10^{-3} M did not either show any division figure or allow mitosis to undergo normal course. In view of no observable spindle microtubules, their action may primarily on the dissolution of the microtubules. In contrast to the four herbicides, chlorpropham (Plate 8), karbutylate (Plates 18, 19), propham (Plate 22), and simazine (plate 24) acted to disorient spindle microtubules, thus resulting in abnormal cell plate formation. All these four herbicides acted at the concentration range of 10^{-4} to 10^{-5} M. It is of interest that chlorpropham and trifluralin acted to reduce the number of cell wall associated microtubules (Plate 28 for trifluralin effect).

Excessive vacuolation of cytoplasm was caused by atrazine at 10^{-3} M (Plate 5), diquat at 10^{-3} M (Plate 12), and DNOC at 10^{-4} and 10^{-5} M (Plates 14, 15). Effects of atrazine and diquat are weak and only observable at 10^{-3} M, causing many small vacuoles. DNOC effect was much more pronounced with large contorted vacuoles. In closer observations of small vacuoles, it appears that the formation of vacuoles is caused by separation of endoplasmic membranes.

Effects of Herbicides on the Ultrastructure of Soybean Leaf Cells. Similar to the study with corn root tip cells, the morphology of control cells was first examined. Plates 29-32 show water control cells and organelles at three different magnifications. It should be noted that disorganization of vacuolar membrane in Plates 29 and 30 is an artefact due to fixation and sectioning. Chloroplasts are packed with thylakoid membranes and grana stacks. A mitochondrion is seen between adjacent chloroplasts in Plate 31. Again similar to the study with corn root tip cells, 1% DMSO did not cause any alteration in altrastructure of mesophyll cells, but 10% DMSO induced swollen appearance in chloroplasts and mitochondria (Plates 33-35) and somewhat separated thylakoid membranes (Plates 33-35).

Table VII-2 summarizes the results with herbicides. Contrary to the results with corn root tip cells, the ultrastructure of mesophylls of soybean leaves is in general much more resistant to herbicidal effects. Eleven out of 17 herbicides tested showed no effect. They are amitrole, atrazine, bromacil, cacodylate, chlorpropham, diuron, DSMA, karbutylate, monuron, propham, and trifluralin. Of the remaining 6 herbicides, 2,4-D and 2,4,5-T caused massive cell damage and disorganized chloroplasts at 10^{-3} M (Plates 36-38 and 49, 50). This effect is similar to that on corn root tip cells. This drastic effect was not observed at lower concentrations. At 10^{-3} M, diallate caused inflated chloroplasts and mitochondria (Plates 39-41). Note also that thylakoid membranes are more dispersed and disoriented than the 10% DMSO control. Similar observations were made with less concentrations, but with less degrees of distortion. Diquat induced at 10^{-3} and 10^{-4} M somewhat distorted grana

Table VII-2. Summary of effects of 17 herbicides on the ultrastructure of soybean leaf cells.

HERBICIDE	Observed Effect
Amitrole	No effect observed at 10^{-3} M.
Atrazine	No effect observed at 10^{-3} M.
Bromacil	No effect observed at 10^{-3} M
Cacodylate	No effect observed at 10^{-3} M
Chlorpropham	No effect observed at 10^{-3} M
2,4-D	Massive cell damage at 10^{-3} M, but less at 10^{-4} M
Diallate	Enlarged and rounded chloroplasts with somewhat displaced lamellae at 10^{-4} and less so at 10^{-5} M
Diquat	Expanded and/or disoriented lamellae; stroma appears non-uniform perhaps due to lamellar damage; mitochondria appear puckered with enlarged cristae (10^{-3} and 10^{-4} M)
Diuron	No effect at 10^{-4} M
DNOC	Expanded grana in isolated portions of the leaf at 10^{-4} M
DSMA	No effect at 10^{-3} M
Karbutylate	No effect at 10^{-3} M
Monuron	No effect at 10^{-4} M
Propham	No effect at 10^{-3} M
Simazine	Cell and organelle damage at 10^{-4} M; cytoplasm and stroma disrupted throughout the entire cell with more electron dense than usual
2,4,5-T	Massive cell damage at 10^{-3} M, but not at 10^{-4} M
Trifluralin	No effect at 10^{-3} M

stacks and thylakoid membrane (Plates 42 and 44). Mitochondria are also affected showing enlarged cristal membrane (Plate 43). Cells and organelles are more electron dense after diquat treatment (Plates 42-44). DNOC caused separation of thylakoid membranes in grana stacks at 10^{-4} M; this observation was frequently limited to isolated portions of the leaf (Plates 45, 46). At 10^{-4} M, simazine induced disorientation of thylakoid membranes and disruption of cytoplasm and stroma (Plates 47 and 48). Similar to diquat treatment, simazine acted to increase electron density of cytoplasm and chloroplasts.

Comparison of herbicidal effect on alteration of ultrastructure of root tip cells and that of soybean leaf cells reveals that (a) leaf cells are more resistant to herbicides than root tip cells and (b) with the exception of 2,4-D and 2,4,5-T effects, the effective herbicides on both types of cells acted differently. Since root tip cells are undifferentiated, young developing cells and leaf cells are mature, fully differentiated cells, inhibitors such as herbicides may cause more pronounced effects on young, developing cells than on mature, fully differentiated cells. The difference in cell types may be responsible for different effects. 2,4-D and 2,4,5-T, however, must be strong agents at high concentrations to cause the same basic effect on the two types of cells.

DISCUSSION

With the treatment period of 2 hours, we observed structural alteration in root tip cells of mitotic figure, cell plate formation, mitochondria, plastids, cell wall, vacuolation, and general cell appearance. Herbicides are not effective in causing abnormalities in the structure of nuclei, golgi apparatus, and ribosomes (see Anderson and Thomson, 1973). Mitochondria are the most susceptible organelle, and mitosis and cell plate formation are the most susceptible process (Table VII-1). Since the effect on mitosis and cell plate formation is mediated by herbicidal action on spindle microtubules, orientation and/or dissolution of the microtubules are susceptible to herbicide treatments (Bartels and Hilton, 1973; Hepler and Jackson, 1969). The effect of herbicides on the cell wall or associated microtubules is in general small, perhaps because these structures are more stable ones than mitochondria and mitotic microtubules (Table VII-1). The susceptibility of mitochondria and spindle microtubules may further indicate that these organelles are needed in the metabolic activity and differentiation processes of root tip cells. Another observation that excessive vacuolation is caused by three herbicides is difficult to explain. However, the formation of small vacuoles is apparently originated at the endoplasmic reticulum. Separation of the membranes of the endoplasmic reticulum and subsequent filling of the holes with the cell sap must take place. Since DNOC is a potent uncoupler of oxidative phosphorylation of isolated mitochondria (CHAPTER II) and it may act to lower the cellular concentration of ATP (cf. Gruenhagen and Moreland, 1971), atrazine and diallate which cause excessive vacuolation may function by interrupting the cellular energy metabolism (cf. Fig. II-17).

Since the main function of mesophyll cells is to carry out photosynthesis, the action of 4 effective herbicides on alteration of chloroplast structure can be anticipated from the analogy with herbicidal effect on root cell structure. Mitochondria in leaf cells appear not to be susceptible to herbicides (Anderson and Thomson, 1973). In general, the chloroplast structure is frequently altered by herbicidal application to seedlings and to photosynthetic tissues (Anderson and Schaelling, 1970; Anderson and Thomson, 1973; Ashton et al., 1963; Bartels, 1965; Hallam, 1970; Harris and Dodge, 1972).

Drastic effects caused by 2,4-D and 2,4,5-T at mM concentrations have been reported (Hallam, 1970). It should be noted that the effects are only seen at high concentrations; this point alone casts doubt as to whether the primary mode of these phenoxy herbicidal action is in massive destruction of plant cell structures (see Anderson and Thomson, 1973).

Subcellular morphology of root tip cells is highly susceptible to herbicides, while herbicides caused relatively weak effects on the respiratory activity and the syntheses of protein and nucleic acids in corn root tips. Morphological observations thus appear to be more sensitive than physiological studies. With soybean leaf cells, the situation reverses; leaf cells are relatively insensitive to morphological alteration, but highly sensitive to physiological changes. These comparisons strongly suggest that morphological and physiological analyses are highly useful in assessing herbicide phytotoxicity.

EVALUATION OF METHOD

Herbicidal action on root tip cells appears to be more sensitively detected by ultrastructural observations than by physiological analyses. With a photosynthetic tissue which is fully mature and well differentiated herbicidal action can be more readily detected by physiological methods than by ultrastructural observations. It appears that the organelles which are involved in the major function of a tissue tend to be susceptible to herbicidal alteration of their ultrastructure. If such organelle activity is not detected by conventional, physiological techniques (e.g., activity of spindle microtubules), electron microscopic studies provide a powerful means of assessing herbicide phytotoxicity.

Although the preparatory method we employed in this study is time-consuming, improvements of electron microscopic techniques are possible.

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EXPLANATION OF PLATES (ELECTRON PHOTOMICROGRAPHS)

Plate 1. Cell plate formation and spindle microtubules in control (water treated) corn root tip cells. x 10,600.

Plate 2. Mitochondria, cell wall, and wall microtubules in control (water treated) corn root tip cells. x 33,250.

Plate 3. Swollen mitochondria, showing the effect of DMSO treatment (10%), in a corn root tip cell. X22,600.

Plate 4. Somewhat beady cell wall in corn root tip cells, showing an effect of 10^{-3} M amitrole. x 33,250.

Plate 5. Excessive vacuolation due to inflated endoplasmic reticulum, resulting from a treatment with 10^{-3} M atrazine. x 9,600.

Plate 6. Expanded, ring-shaped mitochondria in corn root tip cells after treatment with 10^{-6} M bromacil. x 9,600.

Plate 7. Elongated mitochondria in a corn root tip cells after treatment with 10^{-3} M cacodylate. x 13,000.

Plate 8. Abnormal cell plate formation in corn root tip cells due to treatment with 10^{-4} M chlorpropham. x 33,250.

Plate 9. Extensive cell damage in corn root tip cells after treatment with 10^{-3} M 2,4-D. x 2,000.

Plate 10. Massive cell damage in corn root tip cells after treatment with 10^{-3} M 2,4-D. x 2,000.

Plate 11. Lack of mitotic figures and occasional invagination of plasma membrane in corn root tip cells after treatment with 10^{-3} M diallate. x 5,850.

Plate 12. Excessive vacuolation in corn root tip cells due to treatment with 10^{-3} M diquat. x 5,850.

Plate 13. Elongation of mitochondria and plastids in a corn root tip cell treated with 10^{-4} M diuron. x 21,300.

Plate 14. Extensive vacuolation in corn root tip cells treated with 10^{-4} M DNOC. x 4,000.

Plate 15. Excessive vacuolation and lack of cell plate in a corn root tip cell treated with 10^{-4} M DNOC. x 4,000.

Plate 16. Ring-shaped plastids and somewhat enlarged mitocontria in a corn root tip cell after treatment with 10^{-3} M DMSA. x 17,850.

Plate 17. Swollen mitochondria in a corn root tip cell treated with 10^{-4} M DMSA. x 17,850.

Plate 18. Lack of cell plate formation with transversely oriented spindle microtubules in a corn root tip cell treated with 10^{-3} M karbutylate. x 9,600.

Plate 19. Abnormal cell plate formation with spindle microtubules in corn root tip cells treated with 10^{-3} M karbutylate. x 33,250.

Plate 20. Elongated, abnormal mitochondria in a corn root tip cell treated with 10^{-4} M monuron. x 17,850.

Plate 21. Abnormal plastid in a corn root tip cell treated with 10^{-4} M propham. x 33,250.

Plate 22. Abnormal cell plate formation and ring-shaped mitochondria in a corn root tip cell treated with 10^{-5} M propham. x 9,600.

Plate 23. Elongated abnormal mitochondria in a corn root tip cell treated with 10^{-5} M propham. x 9,600.

Plate 24. Irregular nuclear division and cell plate formation in a corn root tip cell treated with 10^{-3} M simazine. x 9,600.

Plate 25. Abnormal plastids in a corn root tip cell treated with 10^{-4} M 2,4,5-T. x 33,250.

Plate 26. Massive cell damage in corn root tip cells treated with 10^{-3} M 2,4,5-T. x 5,850.

Plate 27. Lack of spindle microtubules between dividing nuclei in a corn root tip cell treated with 10^{-3} M trifluralin. x 45,000.

Plate 28. Fewer microtubules along the cell wall in corn root tip cells treated with 10^{-3} M trifluralin. x 45,000.

Plate 29. Mesophyll cells in water-infiltrated soybean leaf. x 3,750.

Plate 30. A mesophyll cell in water-infiltrated soybean leaf. x 5,500.

Plate 31. Chloroplasts in a mesophyll cell of water-infiltrated soybean leaf. x 16,750.

Plate 32. Chloroplasts in a mesophyll cell of water-infiltrated soybean leaf. x 16,750.

Plate 33. Somewhat swollen chloroplast in soybean leaf mesophyll infiltrated with 10% DMSO. x 21,250.

Plate 34. A mesophyll cell in soybean leaf, showing the effect of infiltration of 10% DMSO. x 12,250.

Plate 35. A mesophyll cell in soybean leaf, showing the effect of infiltration of 10% DMSO. x 12,250.

Plate 36. Disorganized chloroplasts in soybean leaf cell infiltrated with 10^{-3} M 2,4-D. x 21,250.

Plate 37. Massive cell damage in soybean leaf cells treated with 10^{-3} M 2,4-D. x 5,500.

Plate 38. Disorganized chloroplasts in soybean mesophyll cells treated with 10^{-4} M 2,4-D. x 16,750.

Plate 39. Enlarged, swollen chloroplasts with somewhat displaced lamellae in soybean mesophyll cells treated with 10^{-3} M diallate. x 17,850.

Plate 40. A close-up view of a chloroplast in soybean leaf cell infiltrated with 10^{-3} M diallate. x 22,600.

Plate 41. Another photomicrograph showing enlarged, swollen chloroplasts in the soybean leaf treated with 10^{-3} M diallate. x 17,850.

Plate 42. Effect of 10^{-3} M diquat on chloroplast structure in soybean mesophyll cells. x 16,750.

Plate 43. Effect of 10^{-4} M diquat on mitochondrial structure in soybean mesophyll cell. x 52,000.

Plate 44. Effect of 10^{-4} M diquat on the ultrastructure of a soybean mesophyll cell. x 16,000.

Plate 45. Expanded grana in a soybean mesophyll cell treated with 10^{-4} M DNOC. x 31,250.

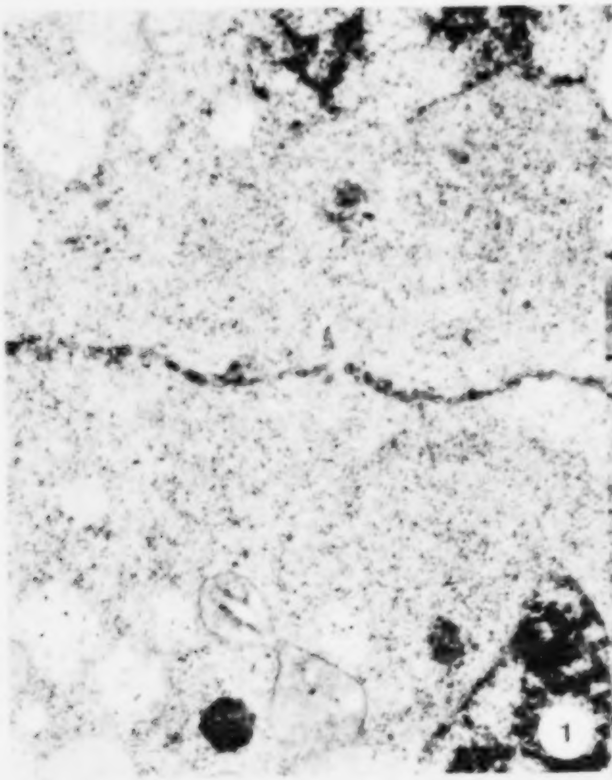
Plate 46. Effect of 10^{-4} M DNOC on chloroplast ultrastructure in soybean leaf. x 16,750.

Plate 47. Effect of 10^{-4} M simazine on ultrastructure of mesophyll cells in soybean leaf. x 5,500.

Plate 48. Disorganized chloroplast in a soybean mesophyll cell treated with 10^{-4} M simazine. x 21,250.

Plate 49. Disorganized chloroplasts in a soybean mesophyll cell treated with 10^{-3} M 2,4,5-T. x 16,750.

Plate 50. Massive cell damage in the soybean leaf infiltrated with 10^{-3} M 2,4,5-T. x 9,000.



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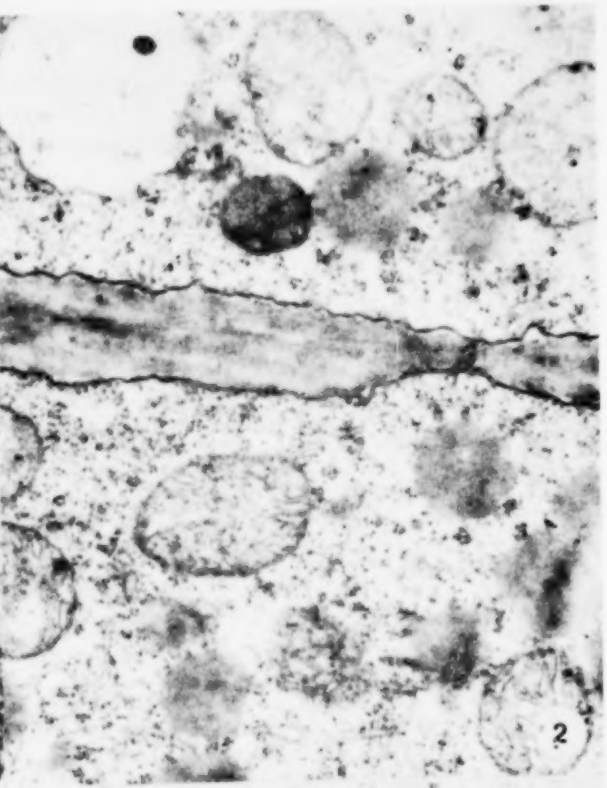

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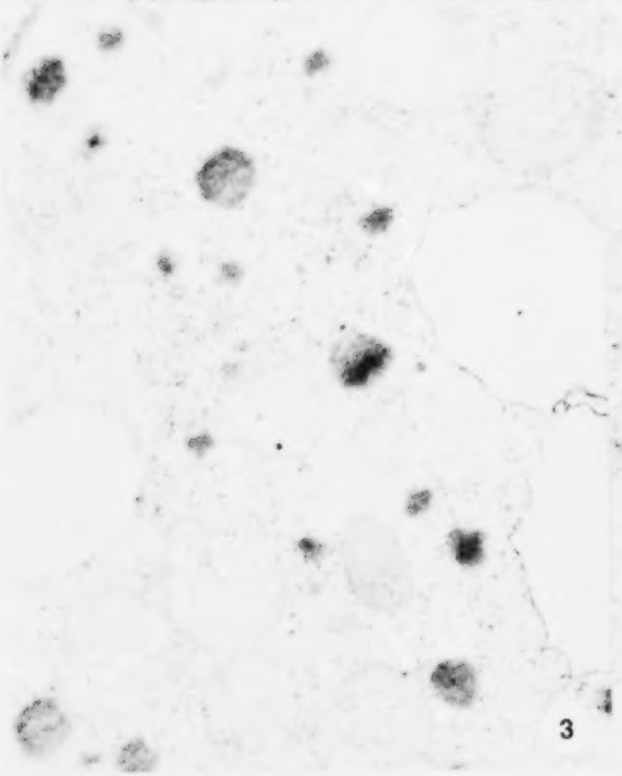

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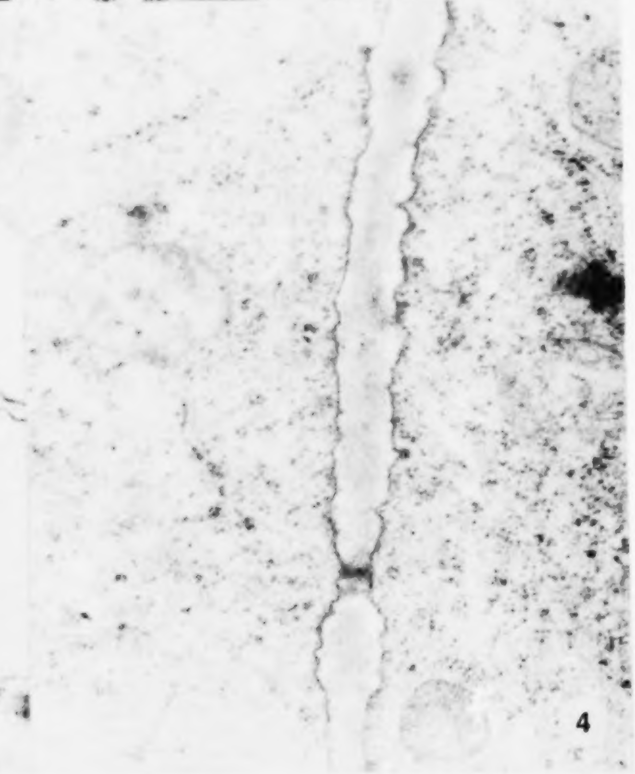

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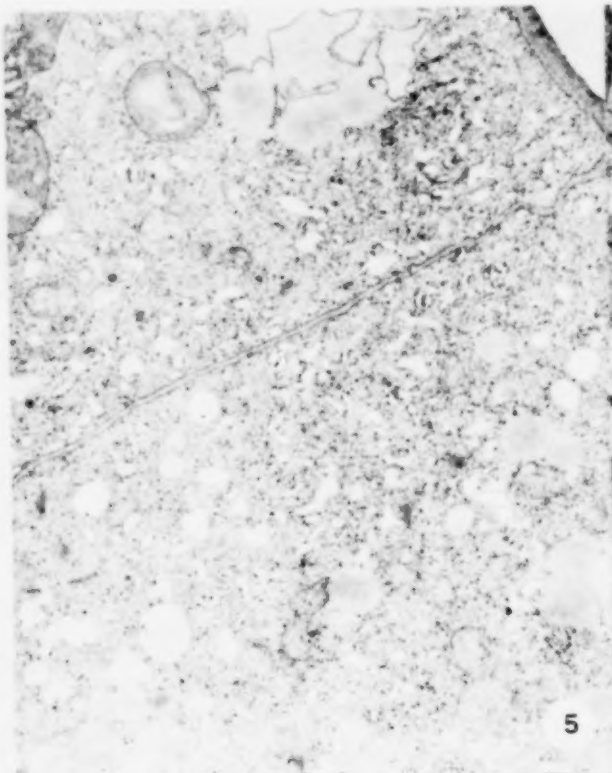

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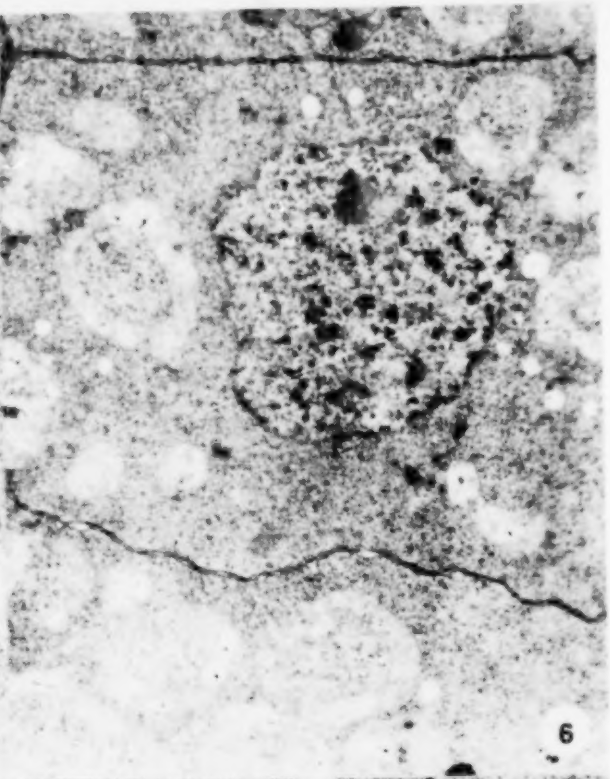

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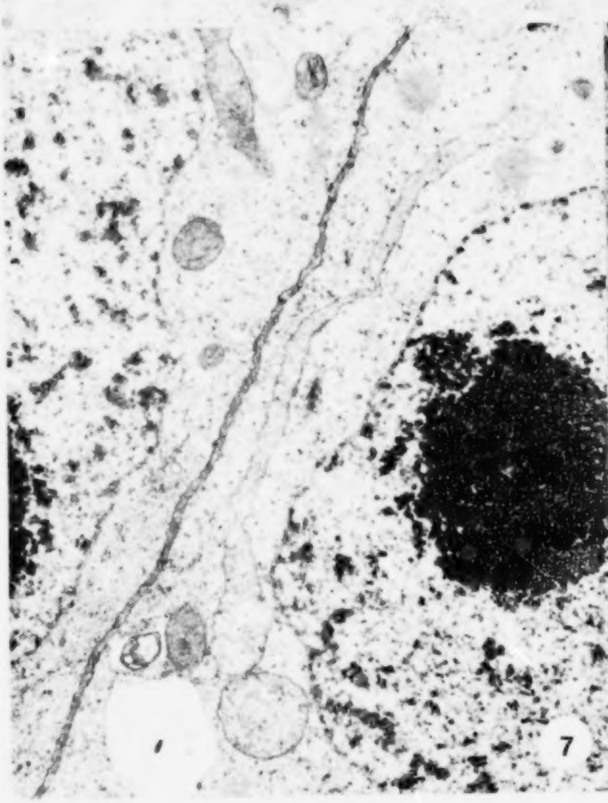

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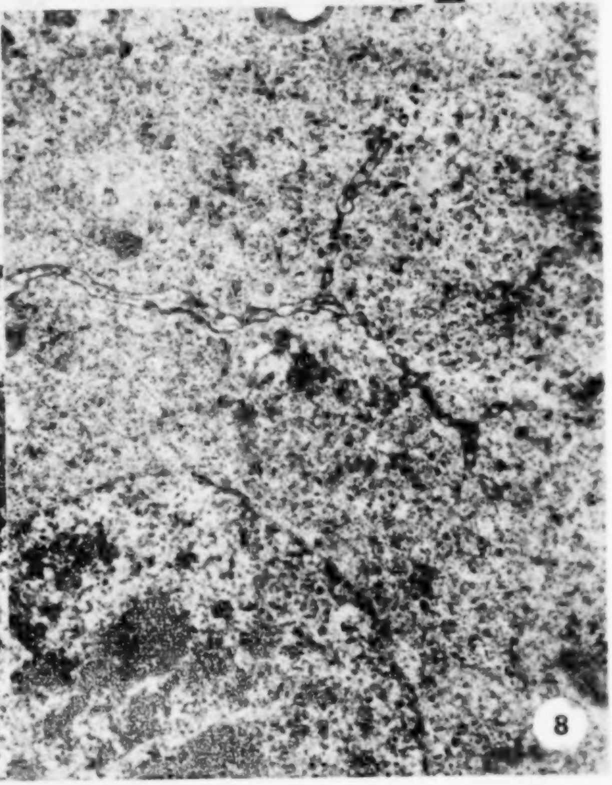

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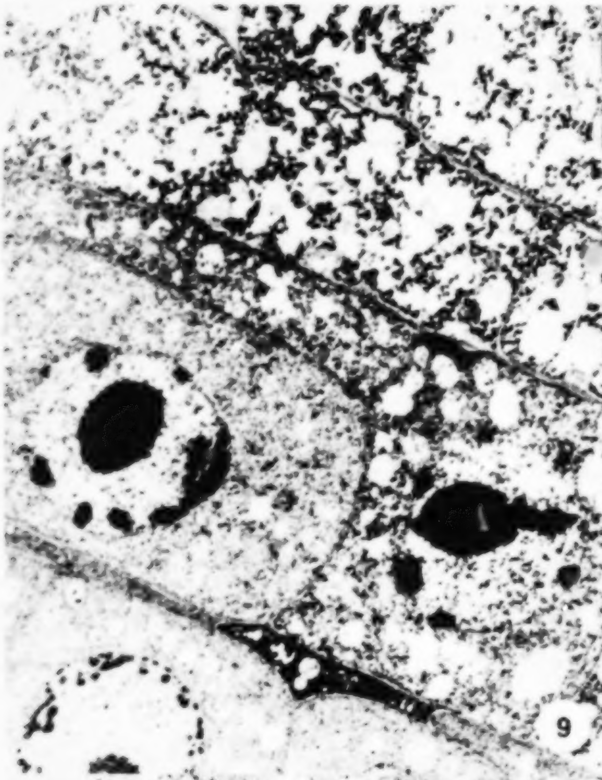

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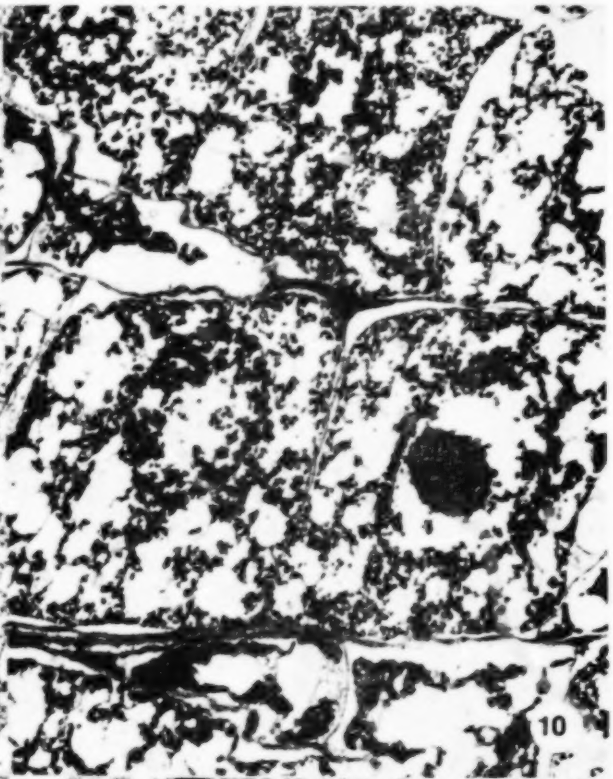

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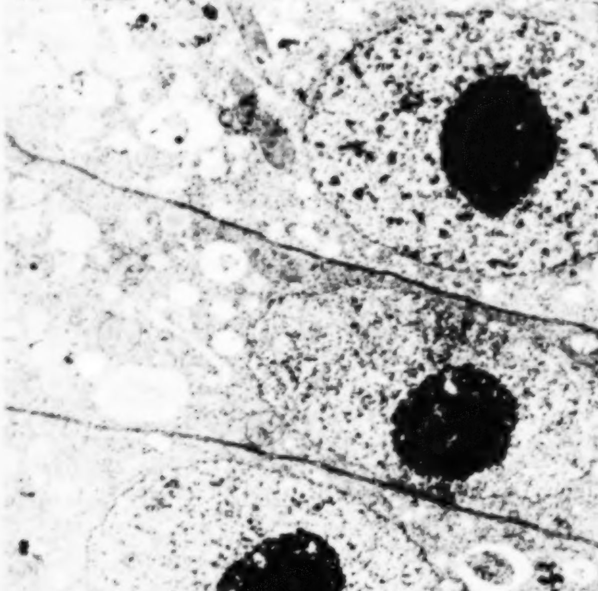

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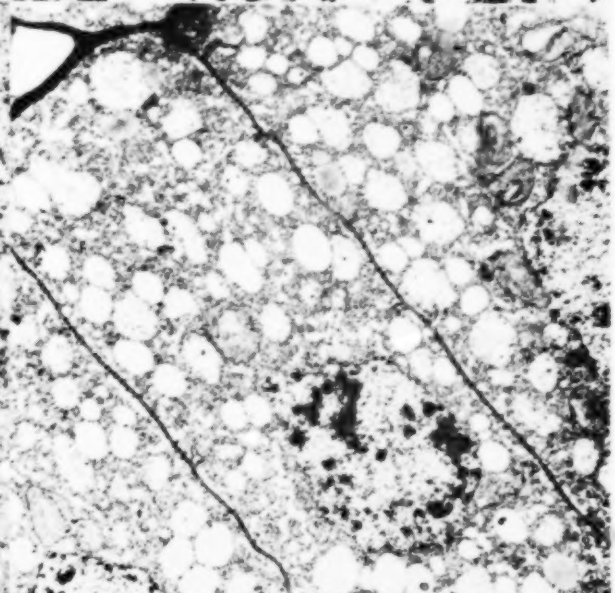

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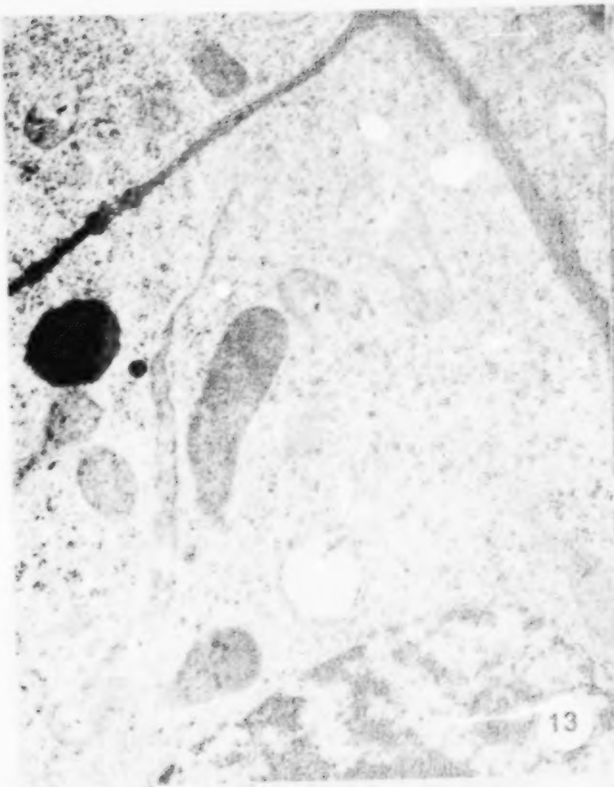

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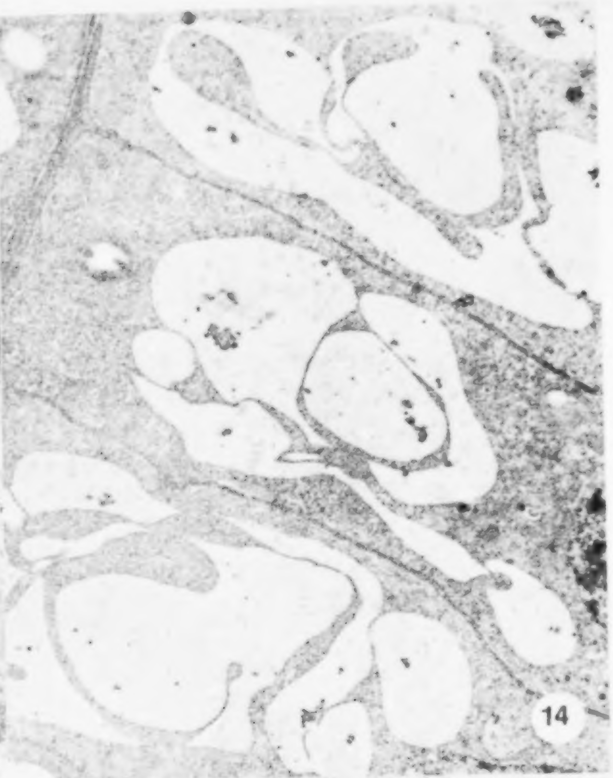

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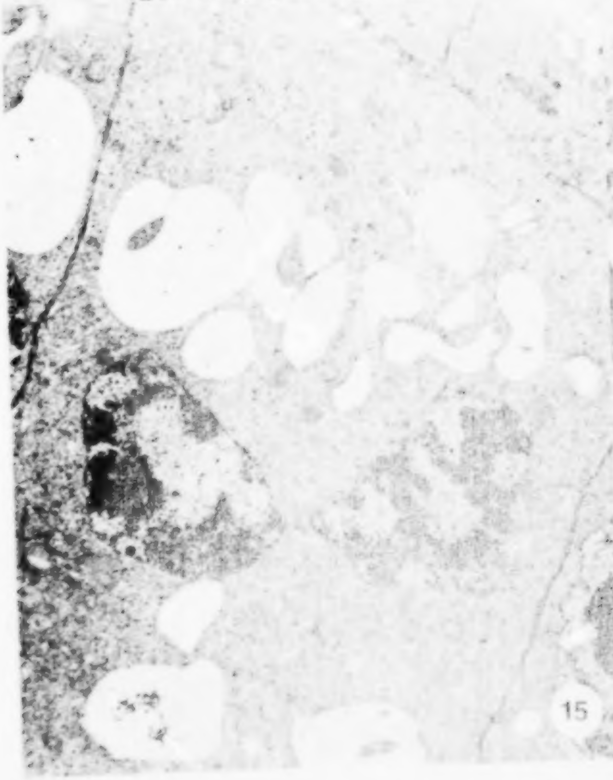

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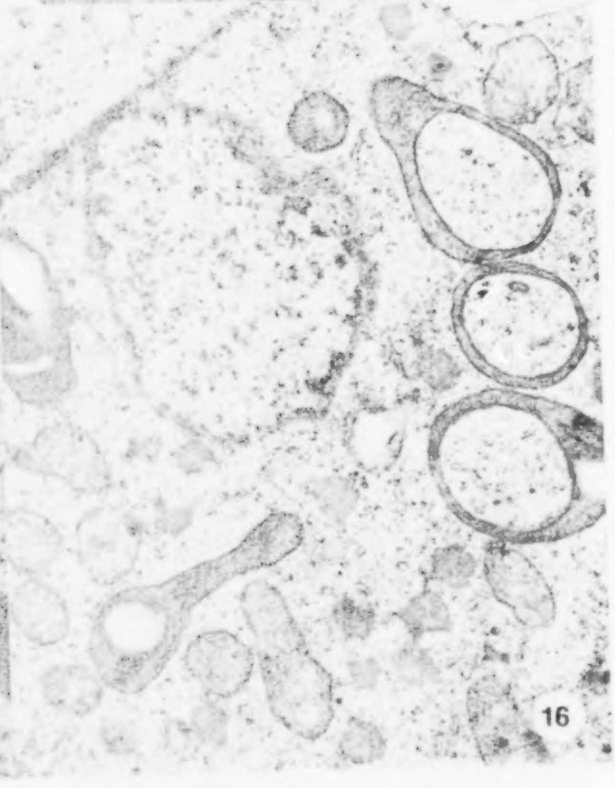

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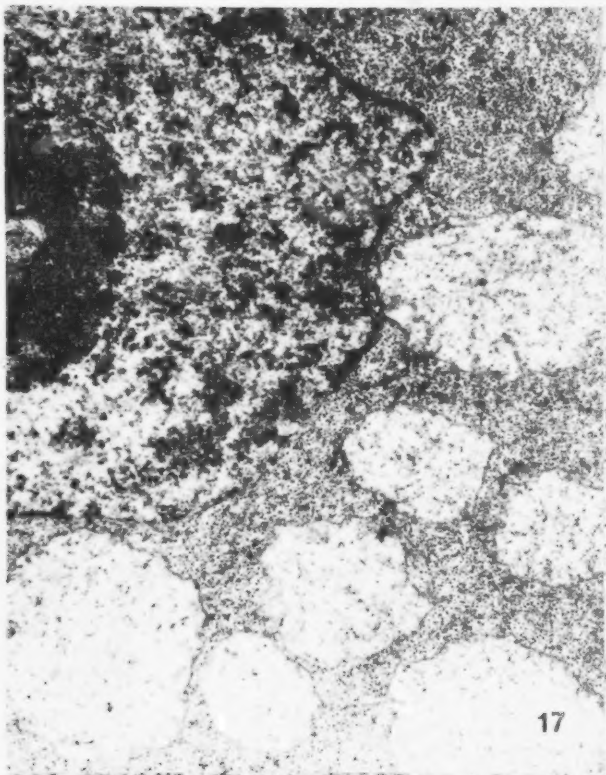

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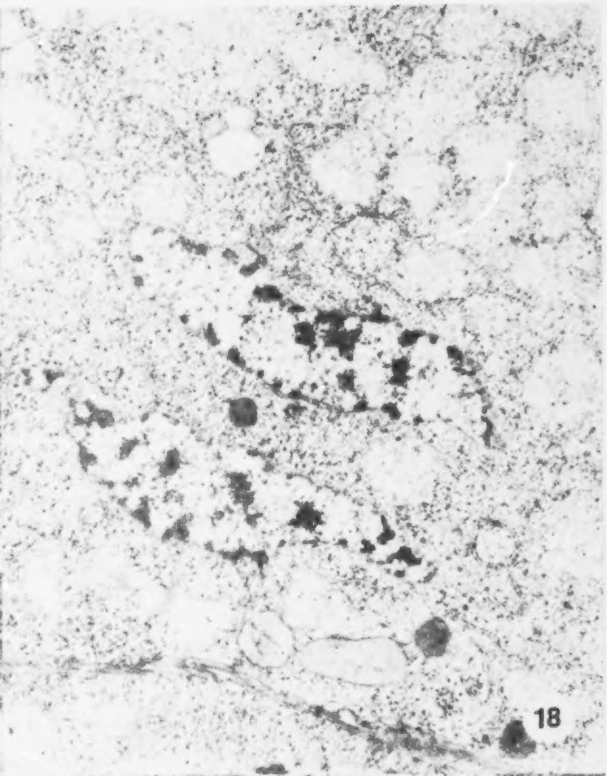

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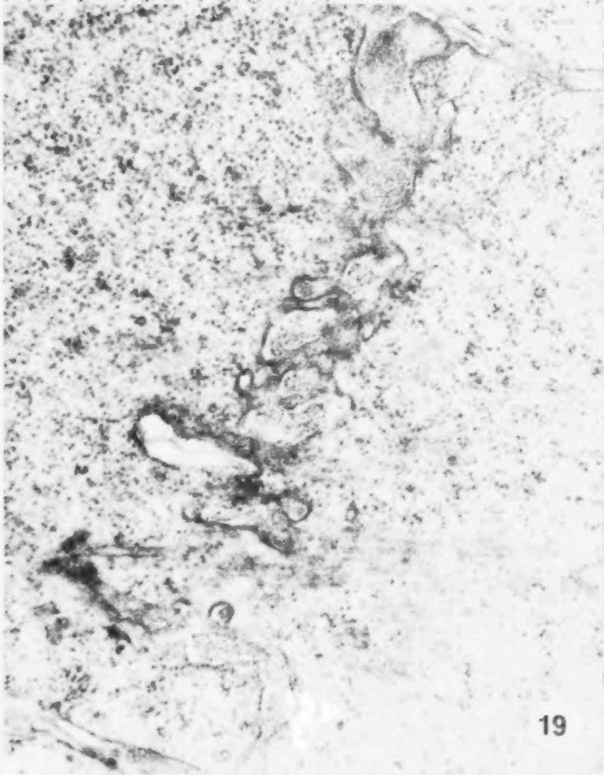

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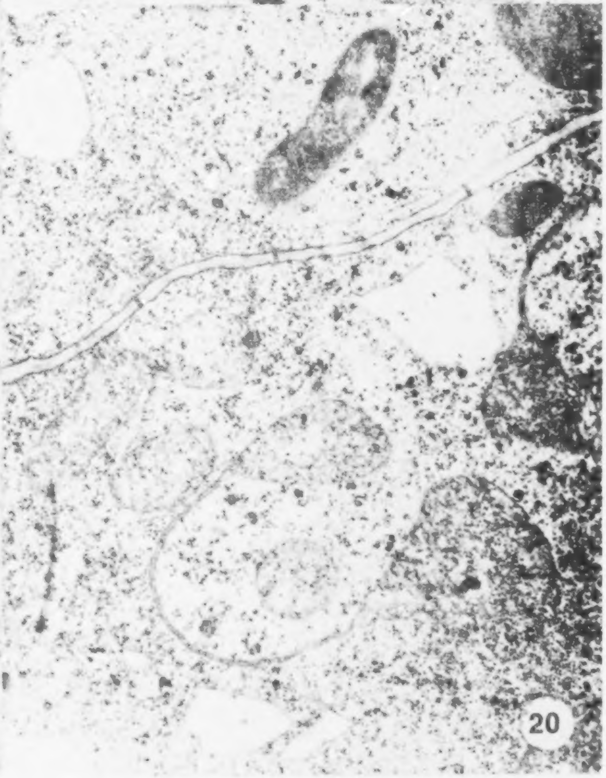

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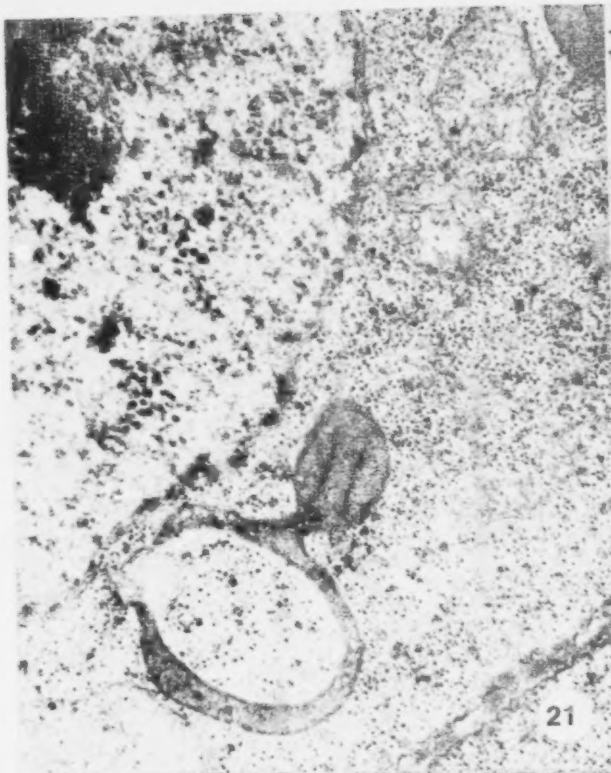
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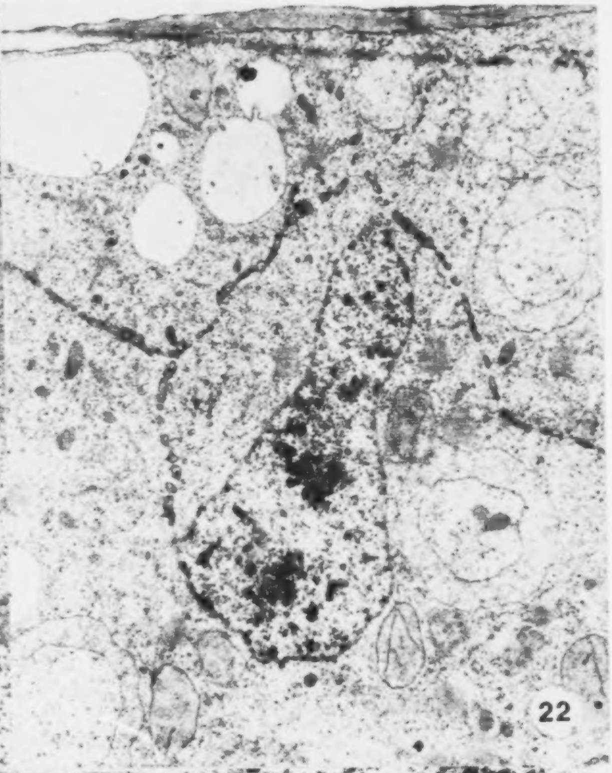
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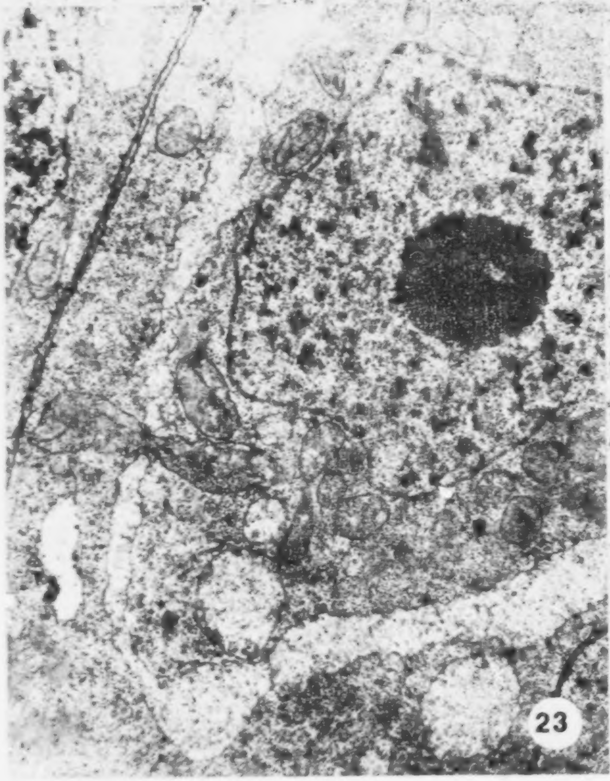
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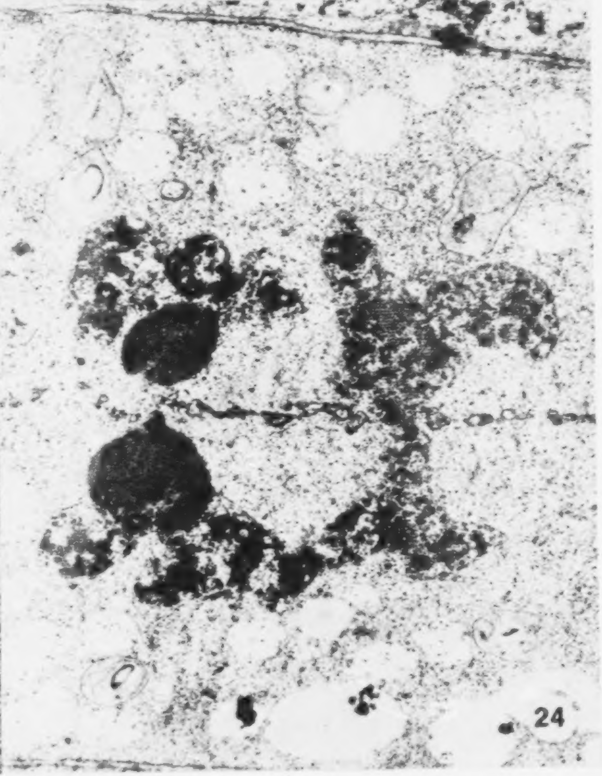
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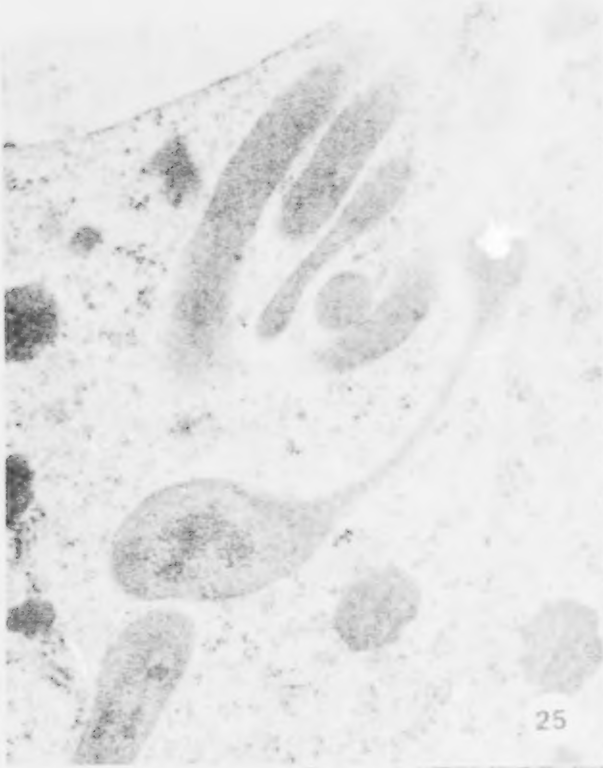
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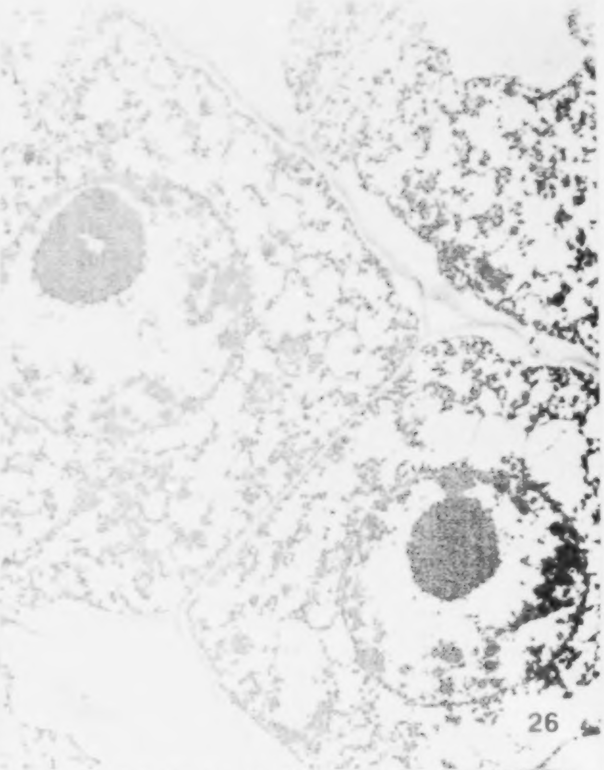
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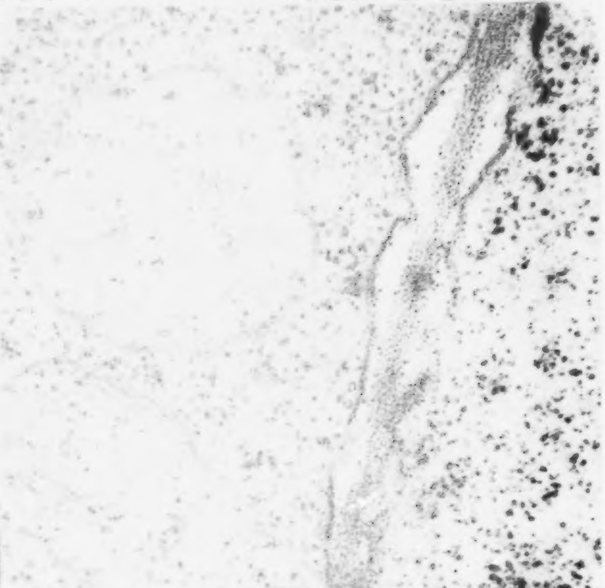
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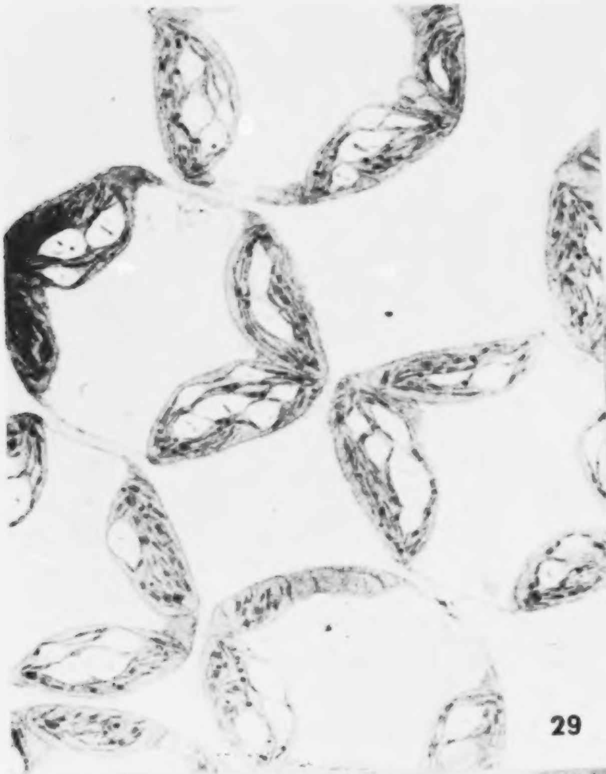

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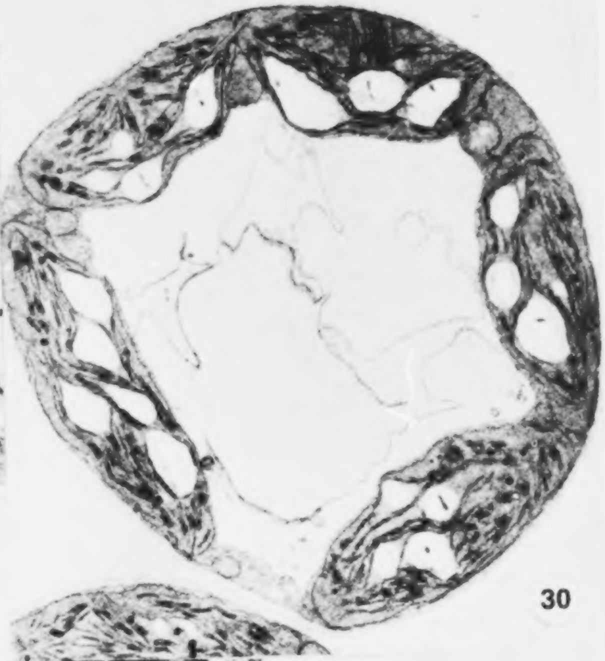

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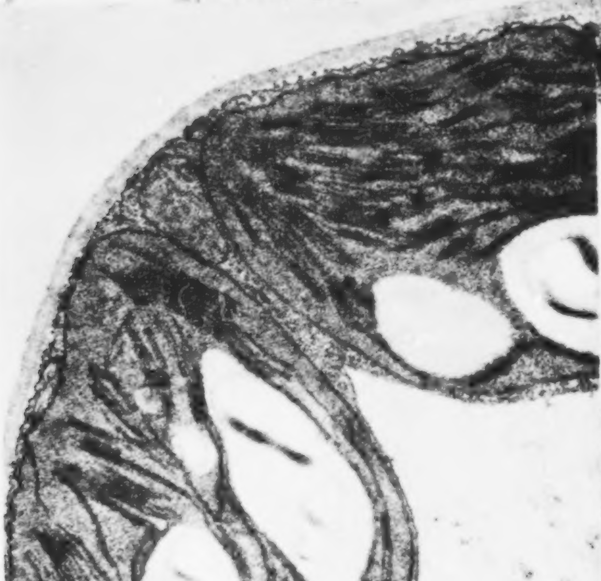

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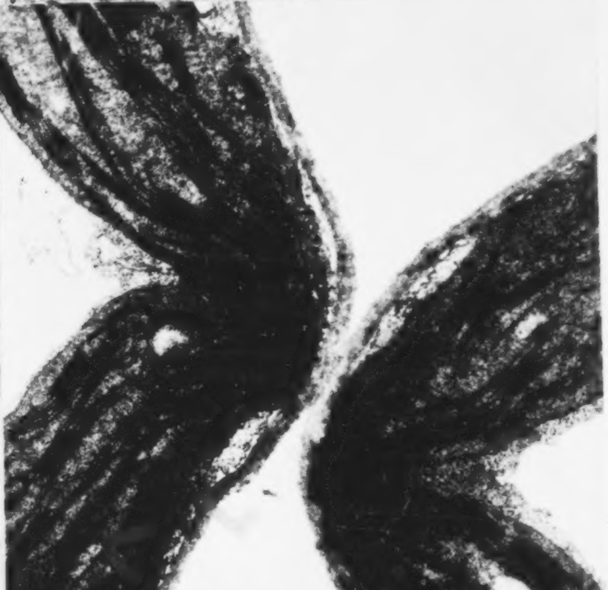

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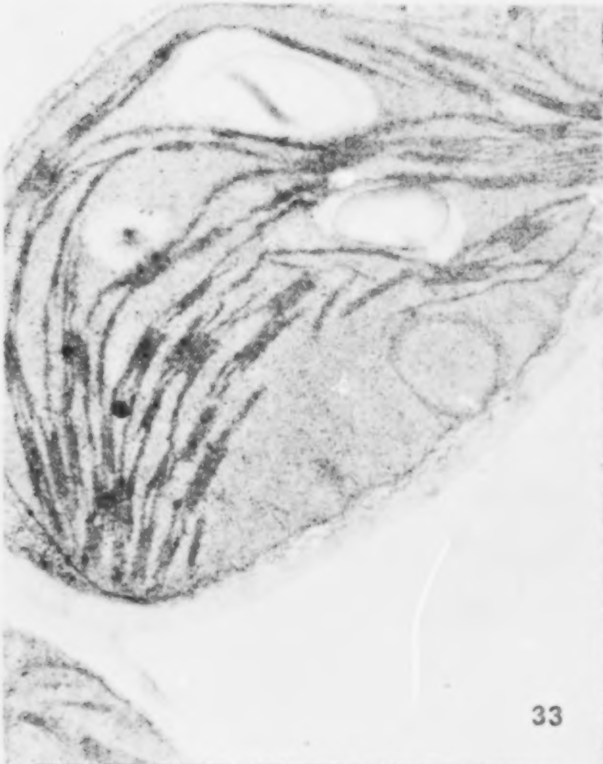
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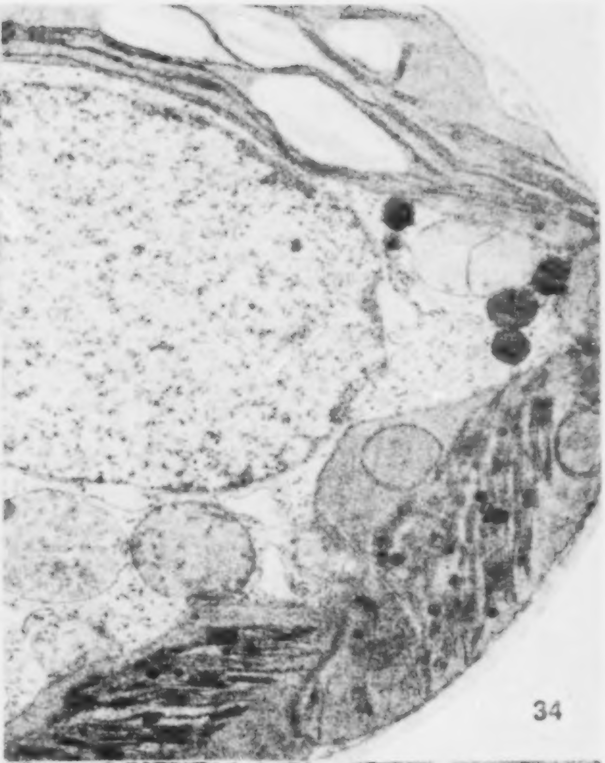
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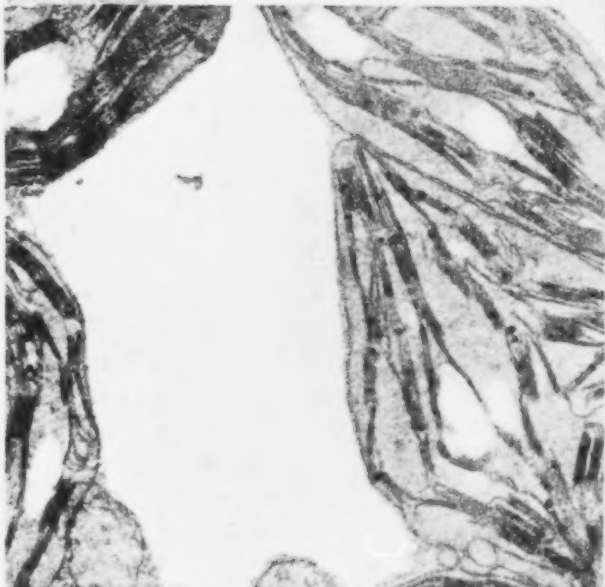
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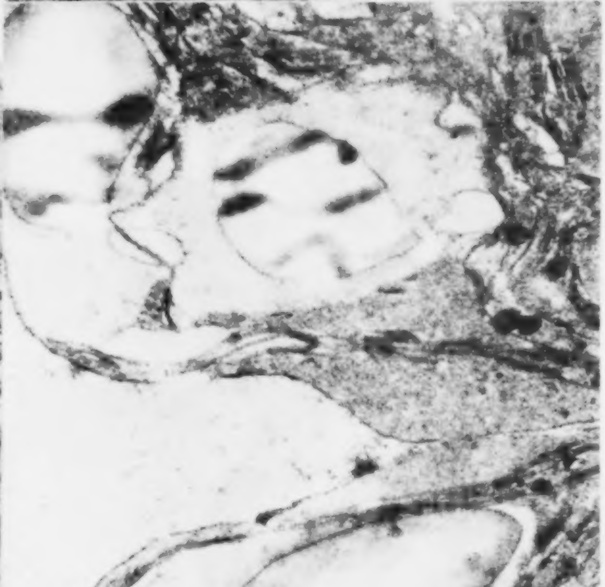
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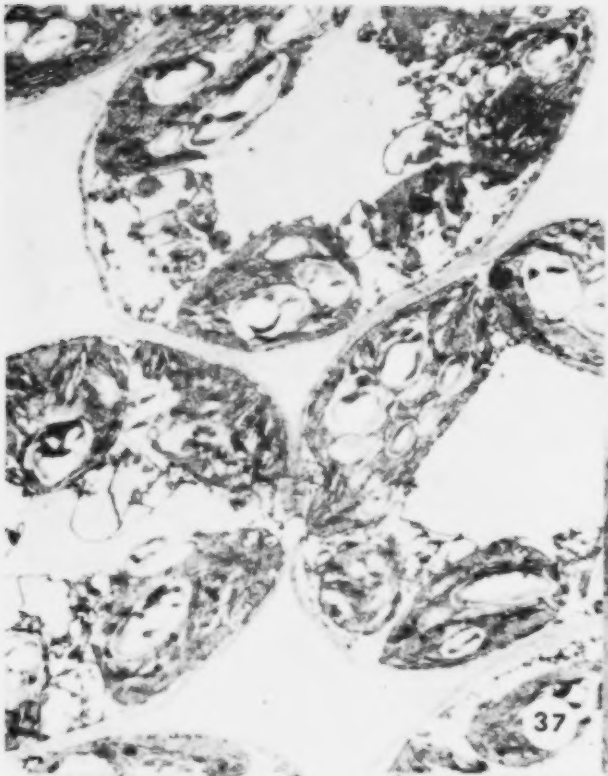
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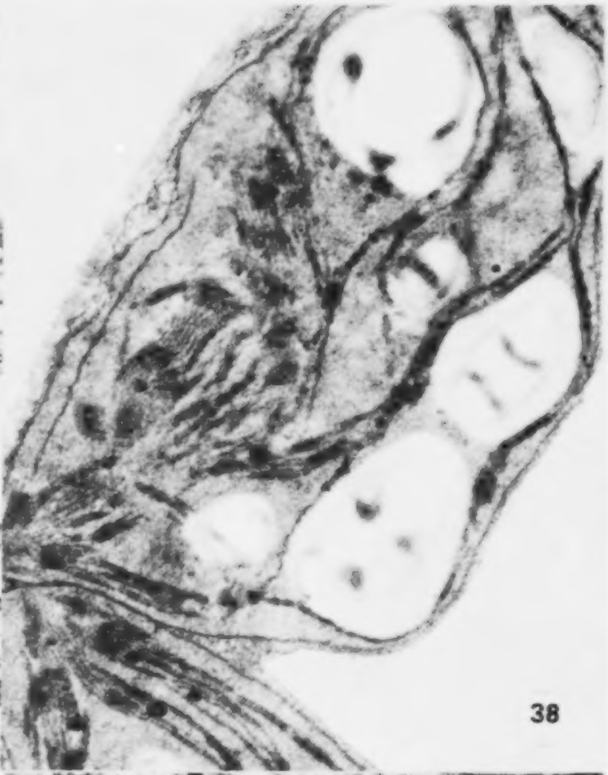
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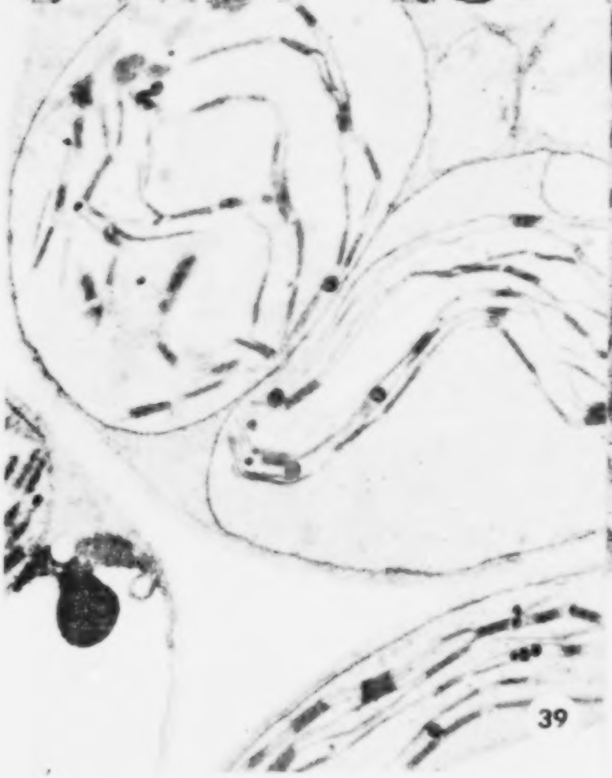
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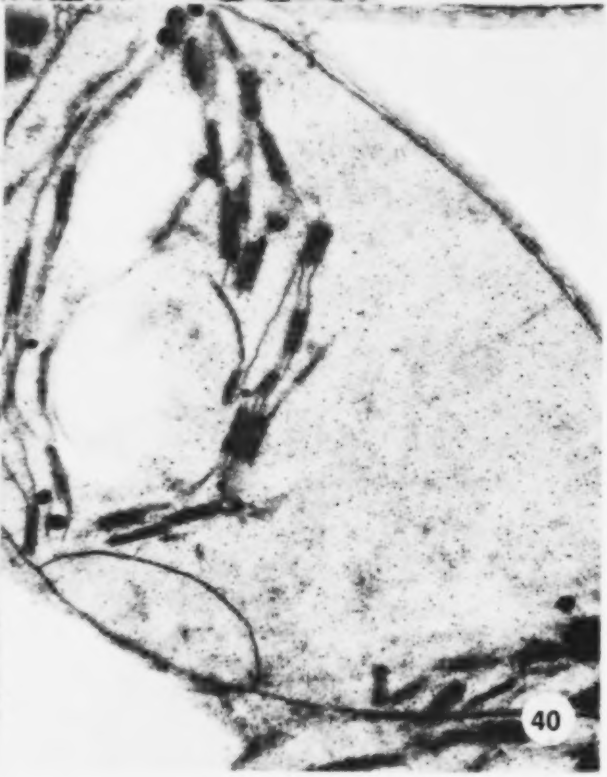
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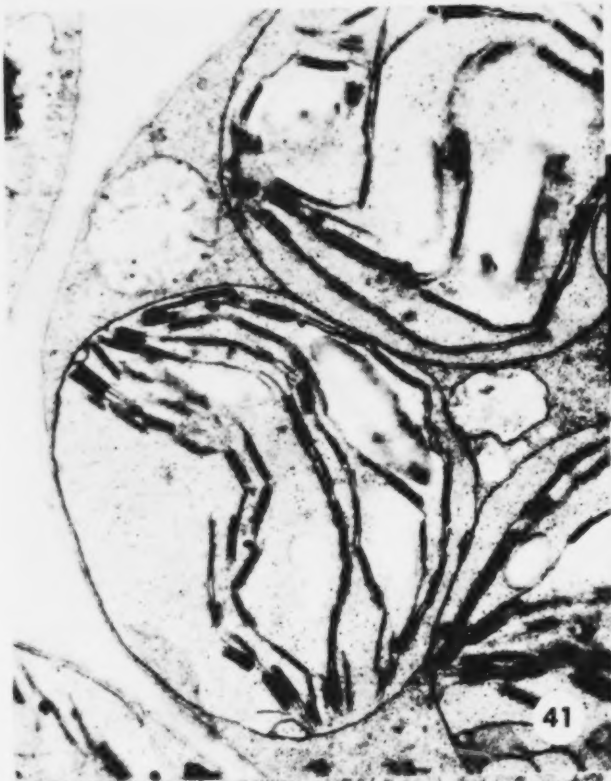
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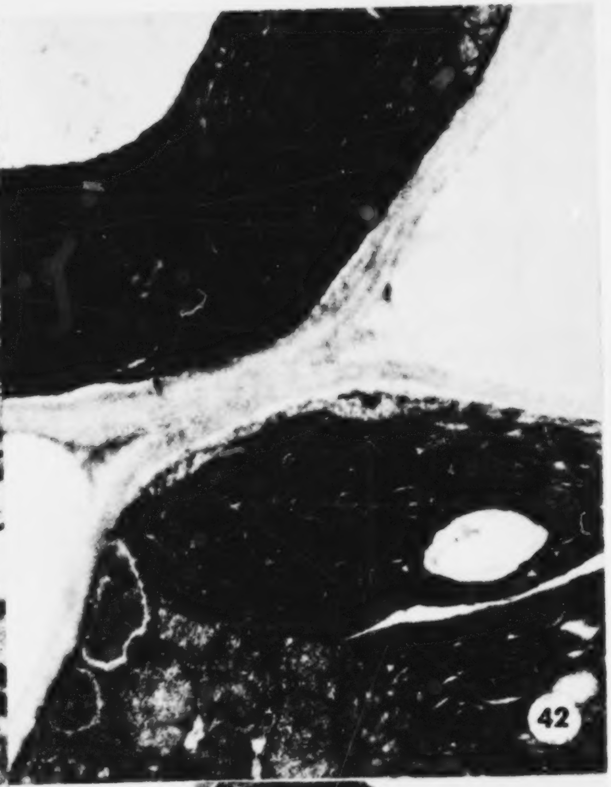
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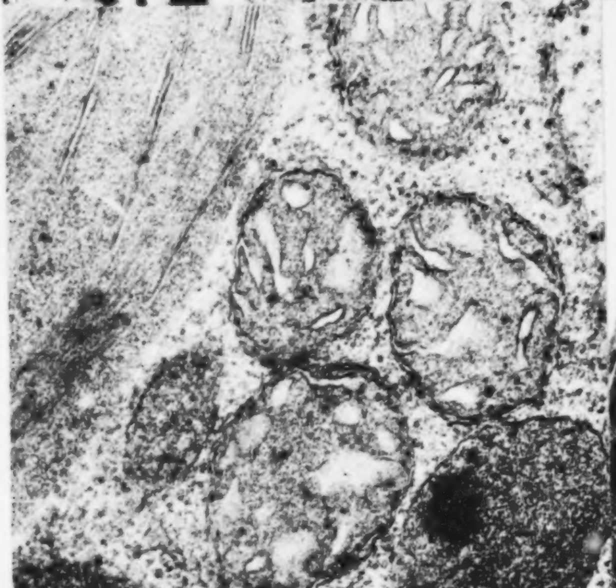
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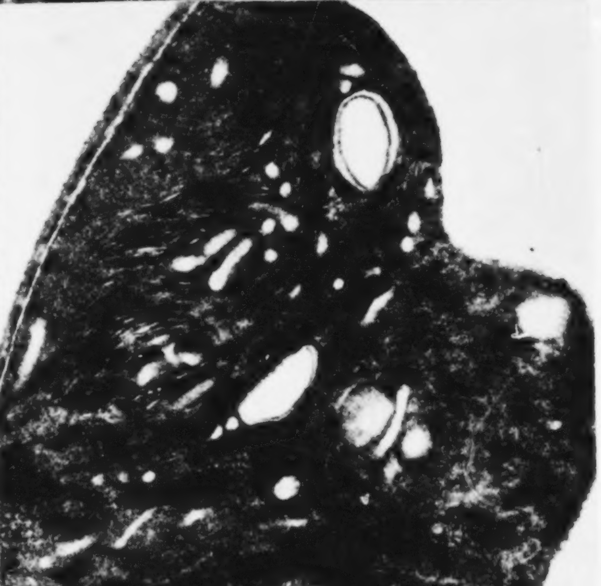
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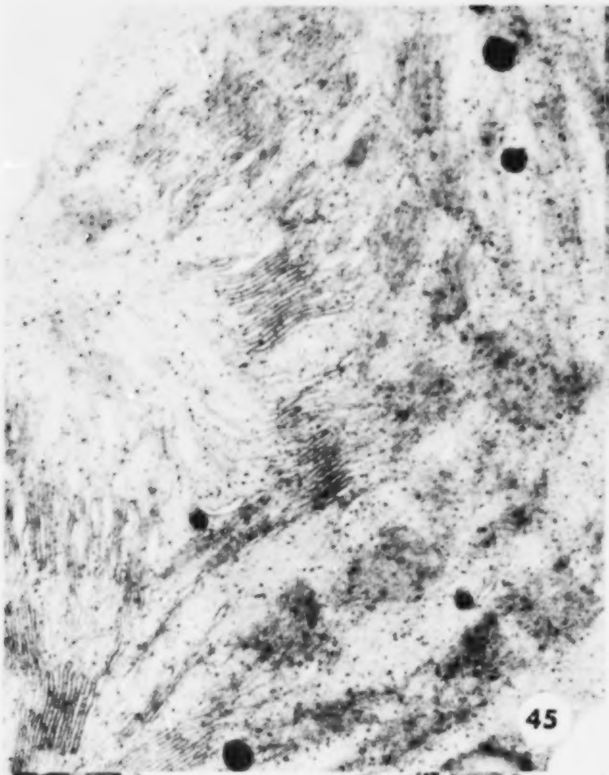
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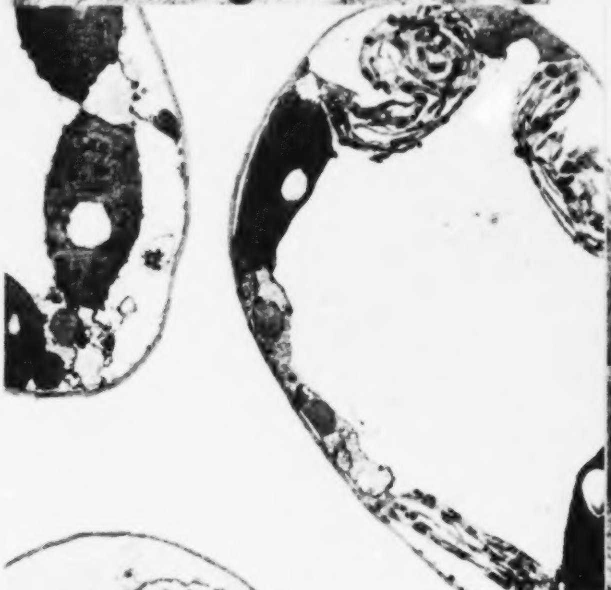
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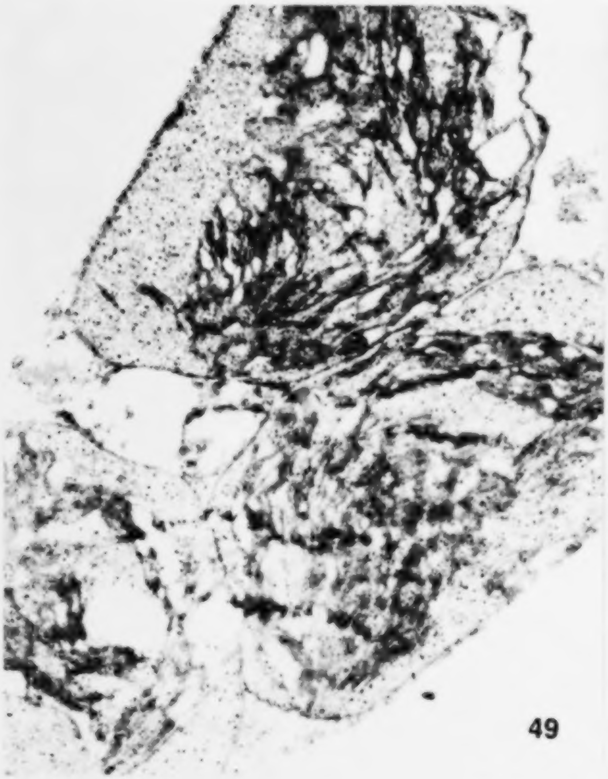

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